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Transcriptional regulation of endothelial cell-specific *Tie1* and *Vegfr3* genes

Kristiina Iljin

Molecular/Cancer Biology Laboratory
Biomedicum Helsinki and Haartman Institute
University of Helsinki
FINLAND

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SUPERVISOR

Kari Alitalo, M.D., Ph.D.
Research Professor of the Finnish Academy of Sciences
Molecular/Cancer Biology Laboratory
Haartman Institute, Biomedicum Helsinki and
the Helsinki University Central Hospital
University of Helsinki

REVIEWERS

Lea Sistonen, Ph.D.
Professor in Cell and Molecular Biology
Department of Biology
Åbo Academi University

And

Hannu Sariola, M.D., Ph.D.
Professor in Developmental Biology
Institute of Biomedicine
University of Helsinki

OPPONENT

Elisabetta Dejana, Ph.D.
Vascular Biology Laboratory
FIRC Institute of Molecular Oncology
Italy

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CONTENTS

CONTENTS ... **3**

ABBREVIATIONS ... **4**

LIST OF ORIGINAL PUBLICATIONS ... **7**

ABSTRACT ... **8**

REVIEW OF THE LITERATURE ... **9**

1 ENDOTHELIAL RECEPTOR TYROSINE KINASES AND VASCULAR DEVELOPMENT ... **9**

1.1 MOLECULAR STRUCTURE OF ENDOTHELIAL RECEPTOR TYROSINE KINASES AND THEIR LIGANDS ... 9
1.2 CARDIOVASCULAR SYSTEM ... 11
1.2.1 Blood vascular system ... 13
Development of the blood vessels ... 13
Vasculogenesis ... 13
Angiogenesis ... 15
Vascular maturation ... 18
Heterogeneity of the vascular endothelium ... 19
Angiogenesis in the adult ... 20
1.2.2 The lymphatic system ... 22
Development of the lymphatic vessels ... 22
Lymphangiogenesis in the adult ... 25

2 REGULATION OF ENDOTHELIAL CELL-SPECIFIC EXPRESSION OF GENES ... **26**

2.1 CHARACTERIZATION OF REGULATORY ELEMENTS OF ENDOTHELIAL SPECIFIC GENES ... 26
2.1.1 The *Tie* genes ... 26
2.1.2 The *VEGFR1* and *VEGFR2* genes ... 28
2.1.3 The *VE-cadherin*, *PECAM-1* and *VWF* genes ... 31
2.1.4 Other genes predominantly expressed in endothelial cells ... 34
2.2 TRANSCRIPTION FACTORS REGULATING ENDOTHELIAL GENE EXPRESSION ... 36
2.2.1 Ets factors ... 36
2.2.2 GATA factors ... 38

3 USE OF ENDOTHELIAL TARGETING ELEMENTS IN FUTURE GENE THERAPY ... **40**

AIMS OF THE PRESENT STUDY ... **42**

MATERIALS AND METHODS ... **43**

RESULTS AND DISCUSSION ... **46**

1. DNA ELEMENTS NEEDED FOR THE *TIE1* PROMOTER ACTIVITY AND CELL TYPE SPECIFICITY (I) ... 46
2. GENERATION OF FLUORESCENT *TIE1* REPORTER MICE TO ALLOW MONITORING OF VASCULAR DEVELOPMENT AND ENDOTHELIAL CELL ISOLATION (II) ... 48
3. THE GENOMIC STRUCTURE AND PROMOTER REGION OF *VEGFR3* (III) ... 50
4. FUTURE PROSPECTS ... 52

CONCLUDING REMARKS ... **54**

ACKNOWLEDGEMENTS ... **56**

REFERENCES ... **57**

ABBREVIATIONS

aa	amino acid
Ang	angiopoietin
AP-2	activator protein-2
bHLH	basic helix-loop-helix
ChIP	chromatin immunoprecipitation
E	embryonic day
EBS	Ets binding site
EC	endothelial cell
ECM	extracellular matrix
EGFP	"enhanced" green fluorescent protein
EGR-1	early growth response transcription factor
Elk-1	Ets-like protein 1
EMSA	electrophoretic mobility shift assay
ENG	endoglin
eNOS	endothelial nitric oxide synthase
EPAS1	endothelial PAS domain protein 1
Erf	Ets2 repressor protein
Erg	transcription factor encoded by Ets-related gene
ET-1	endothelin-1
Ets	group of helix-loop helix transcription factors that contain an ETS DNA-binding domain
FACS	fluorescence activated cell sorting
Flk1	fetal liver kinase 1 (VEGFR-2)
Flt1	fms-like tyrosine kinase 1 (VEGFR-1)
Flt4	fms-like tyrosine kinase 4 (VEGFR-3)
FOXC2	forkhead box C2 transcription factor
GATA1-6	GATA transcription factors 1-6
GFP	green fluorescent protein
HBS	HIF binding site
hGH	human growth hormone
HIF	hypoxia-inducible factor
hnRNA	heterogenous nuclear RNA
HR	homology region
HSPG	heparan sulfate proteoglycan
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
kbp	kilobasepair
KDR	kinase insert domain containing receptor (VEGFR-2)
LacZ	bacterial gene encoding for ß-galactosidase enzyme
LKLF	lung Kruppel-like factor
LLC	Lewis lung carcinoma
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
MAPK	mitogen-activated protein kinase
MEF-2	myocyte enhancer factor 2
MMP	matrix metalloproteinase
mRNA	messenger RNA
Net	new Ets transcription factor
Oct1	ubiquitous octamer-binding protein
P	postnatal day
PAS	Per/Arnt/Sim
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PECAM	platelet-endothelial cell adhesion molecule

NERF	new ets related factor
NF-1	nuclear factor-1
PI3K	phosphatidylinositol 3′-kinase
PlGF	placenta growth factor
PMA	phorbol 12-myristate 13-acetate
Prox-1	prospero-related homeobox protein 1
RTK	receptor tyrosine kinase
SAP-1	serum response factor accessory protein 1
Scl/Tal	Stem cell leukemia/T-cell acute leukemia
SLC	secondary lymphoid organ chemokine
SMC	smooth muscle cell
Sp1	specificity protein 1, transcription factor which binds to GC box element
Tek	tunica interna endothelial cell kinase (Tie2)
TGF	transforming growth factor
Tie	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
TNFα	tumor necrosis factor-α
VE-cadherin	vascular endothelial-cadherin
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
Vezf1	vascular endothelial zinc finger 1
vWF	von Willebrand factor
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Iljin, K., Dube, A., Kontusaari, S., Korhonen, J., Lahtinen, I., Oettgen, P., and Alitalo, K.: Role of Ets factors in the activity and endothelial cell specificity of the mouse Tie gene promoter. FASEB J., 13, 377-386, 1999.

II Iljin, K., Petrova, T. V., Veikkola, T., Kumar, V., Poutanen, M., and Alitalo, K.: A fluorescent *Tie1* reporter allows monitoring of vascular development and endothelial cell isolation from transgenic mouse embryos. FASEB J., 16, 1764-1774, 2002.

III Iljin, K., Karkkainen, M. J., Lawrence, E. C., Kimak, M. A., Uutela, M., Taipale, J., Pajusola, K., Alhonen, L., Halmekytö, M., Finegold, D. N., Ferrell, R. E., and Alitalo, K.: *VEGFR3* gene structure, regulatory region and sequence polymorphisms. FASEB J., 15, 1028-1036, 2001.

ABSTRACT

Endothelial cells lining the blood and lymphatic vessels are dependent on receptor tyrosine kinase mediated signaling, which leads to growth or differentiation responses in the target cells. The endothelial-specific receptor tyrosine kinases can be grouped into two subfamilies: Tie (tyrosine kinase with immunoglobulin and epidermal growth factor like domains) and VEGF (vascular endothelial growth factor) receptors. The Tie family consists of two members, Tie1 and Tie2, whereas three VEGF receptors, VEGFR-1, -2, and -3 have been identified. All of these receptors are vital for the normal development of the blood vasculature.

This study was undertaken to investigate the transcriptional regulation of *Tie1* and *Vegfr3* genes. The *Tie1* promoter region that targets expression of the *LacZ* marker gene specifically to endothelial cells in transgenic mice had been previously identified. We studied *Tie1* promoter activity further and showed that the binding sites for Ets transcription factors are important for its activity. In addition, we demonstrated that several Ets factors transactivate *Tie1* promoter, among which NERF-2 showed the strongest transactivation. Furthermore, we found that the *Tie1* promoter directs the production of high amounts of human growth hormone into the circulation in transgenic mice, indicating that it has potential for use in vectors for endothelial cell-specific gene expression.

To allow intravital monitoring of the vascular development and isolation of primary mouse endothelial cells, we generated fluorescent *Tie1* reporter mice. Although *Tie1* has been used as a universal marker for blood vascular endothelium, our analysis of *Tie1* promoter activity in various reporter mice revealed a significant degree of specificity in different types of endothelial cells. Starting at midgestation, *Tie1* promoter activity became stronger in the arterial than in the venous endothelium. Surprisingly, *Tie1* promoter activity was detected also in the lymphatic vessels. Furthermore, by using a transplantable tumor model, we showed that the regulatory elements needed for *Tie1* expression in tumor neovasculature are not present within the 0.8 kbp mouse *Tie1* promoter, indicating the presence of additional regulatory elements in the *Tie1* locus.

To identify regulatory elements important for lymphatic transcription, we characterized the 5′ flanking regions of human and mouse *VEGFR3* genes. Although *Vegfr3* gene expression is necessary for vascular development, it becomes gradually more restricted to the lymphatic endothelium during embryogenesis. Critical regulatory elements for lymphatic endothelial cell specific activity of the *Vegfr3* promoter *in vivo* were found to reside in a 1.6 kbp fragment upstream of the translational initiation codon. This promoter fragment contains two conserved regions having putative binding motifs for transcription factors belonging to MEF-2, NF-1, AP-2, GATA, Ets and Sp1 families. Further studies are needed to determine which of these binding sites are critical for the *Vegfr3* promoter activity and whether additional enhancer elements exist that are responsible for strong promoter activity in the lymphatic endothelium.

REVIEW OF THE LITERATURE

1 ENDOTHELIAL RECEPTOR TYROSINE KINASES AND VASCULAR DEVELOPMENT

1.1 Molecular structure of endothelial receptor tyrosine kinases and their ligands

Endothelial cells (ECs) lining the blood and lymphatic vessels are dependent on receptor tyrosine kinase (RTK) mediated signaling, which leads to growth or differentiation responses in the target cells. This signaling is required for normal development and maintenance of the vascular bed as well as for angiogenic responses in pathological conditions. RTKs are classified into subclasses based on sequence similarities and distinct structural characteristics. They contain a ligand binding extracellular part, a single membrane spanning region, and a cytosolic domain for signal transduction. A juxtamembrane region is located on the cytoplasmic side and it is followed by the conserved kinase domain. RTKs dimerize in response to the binding of specific ligands. Dimerization is followed by transphosphorylation of the tyrosine residues in the activation loop (Hubbard *et al.*, 1998; Schlessinger, 2000). The phosphorylated tyrosine residues outside the catalytic domain act as specific docking sites for intracellular signaling proteins containing Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, promoting the assembly of intracellular signaling complexes (Schlessinger, 2000). The receptor mediated activation of intracellular signaling cascades usually targets transcription factors, modifies their activity and reprograms gene expression.

Vascular endothelial cells express non-lineage restricted RTKs such as receptors for fibroblast growth factors, epidermal growth factors, and hepatocyte growth factor, but also endothelial cell-restricted RTKs. Two major families of endothelial RTKs consist of Tie (tyrosine kinase with immunoglobulin and epidermal growth factor like domains) and VEGF (vascular endothelial growth factor) receptors (Fig. 1).

Two members of the Tie receptor family, Tie1 and Tie2, have been identified, as well as four Tie2 ligands, called angiopoietins (Angs) 1-4 (Davis *et al.*, 1996; Dumont *et al.*, 1992; Maisonpierre *et al.*, 1997; Partanen *et al.*, 1992; Valenzuela *et al.*, 1999). Although Tie1 was discovered more than ten years ago, no ligand for this receptor has been found. Tie receptors have two immunoglobulin (Ig) homology domains, three fibronectin type III homology domains and three epidermal growth factor homology domains in their extracellular region, followed by a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain interrupted by a small stretch of amino acids commonly referred to as the kinase insert. Ang1 and Ang4 induce the phosphorylation of Tie2, Ang3 acts as an antagonist and the effect of Ang2 is context dependent, either antagonistic or agonistic for the activation of Tie2 (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997; Valenzuela *et al.*, 1999). The structure of angiopoietins can be divided into N-terminal coiled-coil domains and carboxy-terminal fibrinogen-like domains, separated by a linker region. The coiled-coil segment is thought to promote multimerization of the ligand chains. Ang2

appears to exist predominantly as homodimers but it is also capable of forming higher order multimers whereas Ang1 exists predominantly as higher order multimers (Procopio *et al.*, 1999; Davis *et al.,* 2003). The linker region of Ang1 is responsible for its binding to the extracellular matrix (ECM) whereas Ang2 does not bind to the ECM. The fibrinogen-like domain represents the most conserved region of the angiopoietins and is critical for receptor-binding (Procopio *et al.*, 1999).

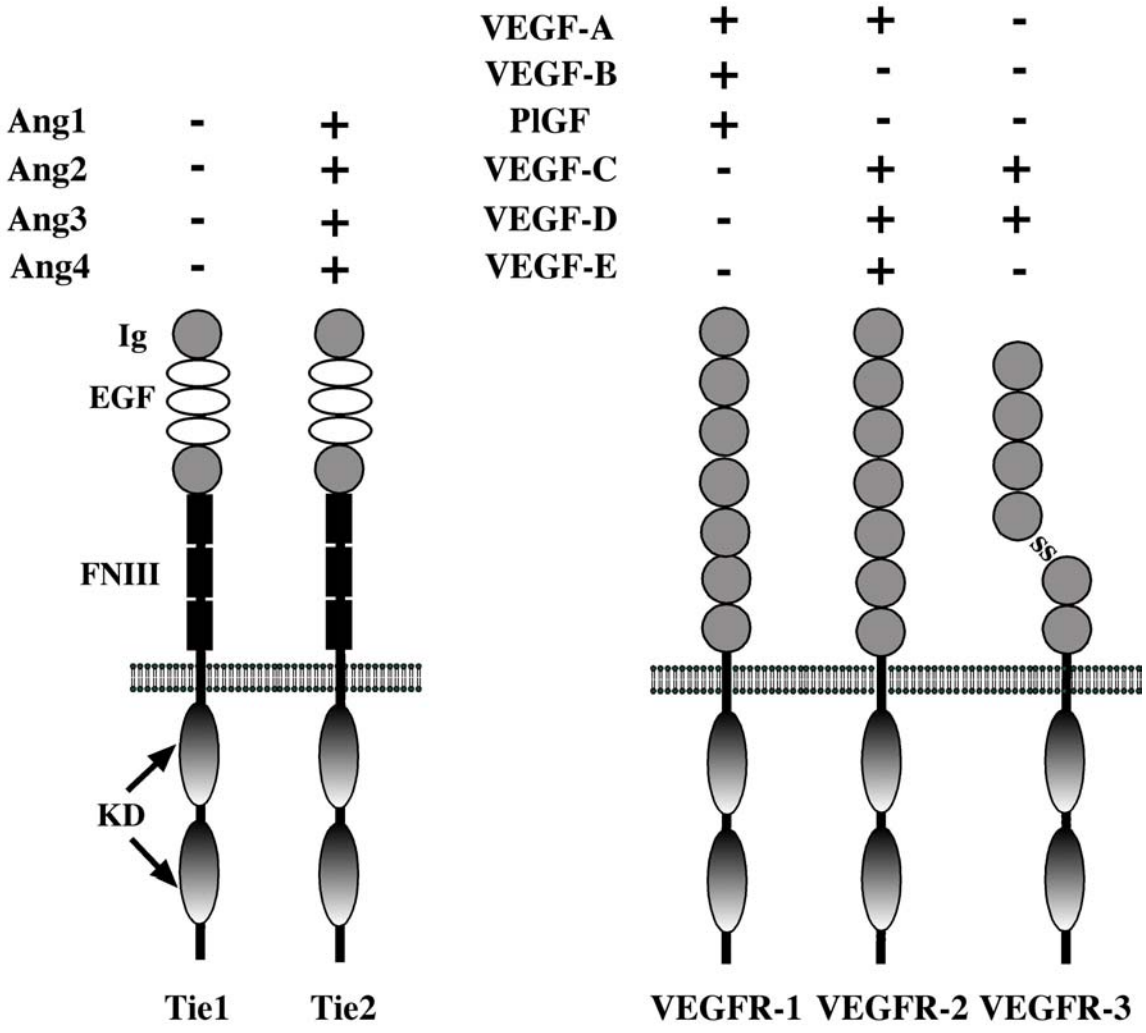

Figure 1. Endothelial cell tyrosine kinase receptors and their ligands. These structurally divergent receptors are subgrouped into the Tie and VEGF receptor (VEGFR) families. Binding specificities of Tie receptor ligands (Ang1-4) and VEGF receptor ligands (VEGF-A to -E and PlGF) are indicated in the figure. Structural motifs present within the receptor families are indicated. Ig, immunoglobulin-like domain; EGF, epidermal growth factor domain; FNIII, fibronectin type III homology domain, KD, kinase domain. Adapted from Jones *et al.*, 2001.

The VEGF receptor family consists of three members, VEGFR-1, -2 and -3, originally named flt1 (fms-like tyrosine kinase-1), KDR (kinase insert domain containing receptor)/FLK-1 (fetal liver kinase-1) and Flt4, respectively. The extracellular regions of VEGFR-1 and VEGFR-2 contain seven Ig domains that are stabilized by disulphide links between paired cysteine residues. In VEGFR-3, the fifth Ig domain is proteolytically cleaved and the resulting polypeptides remain linked by two disulfide bonds (Lee *et al.*, 1996; Pajusola *et al.*, 1993; Pajusola *et al.*, 1992) (Fig. 1). As in Tie receptors, the kinase domain in VEGFRs is split by a kinase insert. Several splice variants have been identified for genes encoding VEGFR-1 and VEGFR-3. In addition to the transmembrane VEGFR-1 splice form, an alternatively spliced form differing in the COOH terminal region, and a soluble splice variant, lacking sequences encoding for the seventh immunoglobulin-like domain as well as transmembrane and intracellular domains, have been characterized (De Vries *et al.*, 1992; Kendall and Thomas, 1993; Shibuya *et al.*, 1990). In humans, a shorter VEGFR-3 transcript, resulting from alternative 3´ polyadenylation signals and lacking the codons for 65 amino acid residues present in the major form has been characterized (Hughes, 2001).

Six growth factors (VEGF-A to -E and PlGF) binding to VEGFRs have been identified. There are several VEGF (VEGF-A) protein isoforms in mouse (120, 164, 188) and in human (121, 145, 165, 183, 189 and 206 aa). All the VEGF isoforms bind to VEGFR-1 and VEGFR-2. VEGF binds to its receptors as a dimer. VEGF may also form heterodimers with VEGF-B and PlGF (placenta growth factor). VEGF-B and PlGF bind only VEGFR-1 whereas VEGF-C and VEGF-D can interact with both VEGFR-2 and VEGFR-3. VEGF-C and VEGF-D are expressed as preproproteins which are proteolytically processed into polypeptides with increasing affinity towards VEGFR-3, and only the fully processed forms can bind to and activate VEGFR-2 (Joukov *et al.*, 1997; Stacker *et al.*, 1999). However, although human VEGF-D is a ligand for both VEGFR-2 and VEGFR-3, mouse VEGF-D binds selectively to VEGFR-3 (Baldwin *et al.*, 2001). Viral VEGF-like polypeptides are commonly named as VEGF-E, and bind to VEGFR-2 (Wise *et al.*, 1999). In addition to the VEGFRs, certain splice isoforms of VEGF, PlGF, VEGF-B, VEGF-C and VEGF-E also bind the semaphorin/collapsin receptors called neuropilins 1 and 2, as well as heparan sulfate proteoglycans (HSPGs) on the cell surface and in the pericellular matrix (Gluzman-Poltorak *et al.*, 2000; Karkkainen *et al.*, 2001; Migdal *et al.*, 1998; Mäkinen *et al.*, 1999; Soker *et al.*, 1998; Wise *et al.*, 1999).

1.2 Cardiovascular system

The cardiovascular system consists of the heart, blood vessels (arteries, veins and capillaries) and lymphatic vessels (Fig. 2). The heart pumps oxygenated blood via arteries to the capillaries where bidirectional exchange of gases and metabolites occurs between blood and tissues. Veins collect deoxygenated blood

from the microvasculature and transport it back to the heart. Due to the high pressure in the circulatory system, fluid leaks out from the blood capillaries. Most of this extravasated tissue fluid is reabsorbed at the venous end of capillaries and postcapillary venules. However, this reabsorption is not sufficient for maintaining body fluid balance, and another system, the lymphatic system, is needed to collect the remaining extravasated tissue fluid. Lymphatic vessels return fluid back to the blood circulation through the thoracic and lymphatic ducts and the lymphaticovenous anastomoses.

The formation of a functional vascular system is the result of a complex series of coordinated processes between different cell types and the ECM. The principal cell types involved in the development of the vascular system are ECs and periendothelial support cells, including vascular smooth muscle cells (SMCs) and pericytes. ECs cover the entire inner surface of blood and lymphatic vessels and form the endocardium, the inner lining of the cardiac lumen. During vascular development, endothelial tubes form first and then acquire the mural cell coating. The ECM between endothelial and periendothelial cells has both structural and signaling functions in vessel formation.

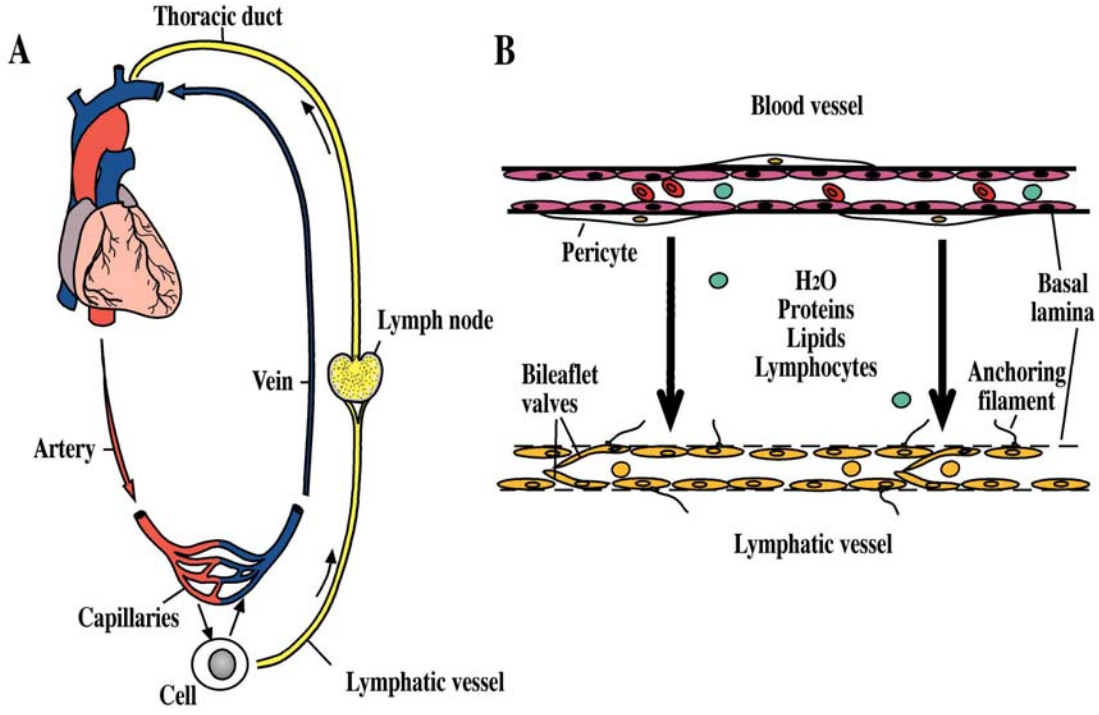

Figure 2. A schematic presentation of the cardiovascular system. (A) Heart, blood vessels and lymphatic vessels form the cardiovascular system. Oxygenated blood is transported via arteries to the capillaries, where bidirectional exchange occurs between blood and tissues. Veins collect deoxygenated blood and carry it back to the heart. Lymphatic vessels collect extravasated tissue fluid, filter it through lymph nodes and return it back to the blood circulation. (B) Blood vessels have a continuous basal lamina with tight interendothelial junctions and they are supported by periendothelial support cells. In contrast, lymphatic vessels have a discontinuous basal lamina and have gaps between the lymphatic ECs. Anchoring filaments attach lymphatic vessels to the surrounding tissue. Bileaflet valves prevent backflow of lymphatic fluid. Adapted from Jones *et al*., 2001.

1.2.1 Blood vascular system

Development of the blood vessels

Cardiovascular system is the first functional organ system to form in order to supply the nutritional requirements of the developing embryo. In humans, development of the circulatory system starts already in the third week of embryonic life. Accordingly, VEGFs and their receptors are expressed early in embryonic development. Results from mRNA *in situ* hybridizations indicate that VEGF and VEGFR-2 expression start in mouse yolk sac and in the embryo proper at embryonic day (E) 7. VEGFR-1 expression starts a bit later, at E7.5 in the amnion and at E9.5 in the embryonic mesoderm. Northern blotting results indicate that VEGF-C expression starts at E7 and by *in situ* hybridization, first VEGFR-3 mRNA expression was detected at E8.5. All VEGFRs have distinct, though partly overlapping expression profiles, suggesting that these RTKs play unique functions in vascularization of the embryo (Kaipainen *et al.*, 1993; Partanen *et al.*, 1999b). Tie1 and Tie2 mRNAs are first expressed around E8 (Dumont *et al.*, 1995; Dumont *et al.*, 1992; Korhonen *et al.*, 1994). In addition to these EC specific receptors, some members of the large Eph RTK family are expressed in ECs and participate in blood vessel development. The significance of signaling via these RTKs to vascular development has been demonstrated using gene-targeted animals. The main processes of vascular development and the molecular mechanisms regulating these events are summarized below and in Table 1.

Vasculogenesis

Hemangioblasts, the progenitor cells of both blood and endothelial cells, differentiate from the embryonic mesoderm. Endothelial progenitor cells are called angioblasts. The formation of blood vessels from *in situ* differentiating angioblasts is called vasculogenesis. In the yolk sac, angioblasts and hematopoietic precursor cells are formed in close association, in so-called blood islands, which are aggregates of hemangioblasts. The inner cells differentiate into hematopoietic precursors and the outer cells into endothelial cells (Fig. 3). Primordial vascular network is formed when blood islands fuse and a lumen is formed by angioblasts (Risau and Flamme, 1995). As a result of subsequent EC proliferation, migration and organization, a primary capillary plexus consisting of homogenously sized endothelial tubes is formed. In the embryo, except for a small region in the aorta, ECs differentiate from the mesoderm as angioblasts, without paralell differentiation of the hematopoietic cells. Intraembryonic hematopoietic precursors localized in the aorta bud serve as founders of lineages of definitive hematopoiesis which occurs sequentially in several organs, including the liver, spleen and bone marrow. The primitive vasculature of endodermally derived organs such as lung, liver, and spleen, heart endocardium and paired dorsal aortas are formed by vasculogenesis.

The molecular mechanisms behind the differentiation of embryonic mesoderm in mouse and in human are still poorly understood. VEGFR-2 is the first molecule known to be expressed in angioblasts and the onset of VEGFR-2 expression in mesodermal precursor cells is thought to mark the establishment of the hemangioblastic lineage during embryonic development (Yamaguchi *et al.*,

1993). Whereas blood islands differentiate spontaneously in a mouse model using embryonic stem cell-derived cystic embryoid bodies, fibroblast growth factors are important for the differentiation of ECs in amphibian and avian models (Flamme *et al.*, 1997; Flamme and Risau, 1992; Mitrani *et al.*, 1990). VEGF secreted by the endoderm may support the differentiation of VEGFR-2 expressing mesodermal cells to angioblasts. Results from knockout studies in mice have proven that VEGF and VEGFR-2 are absolutely critical for the earliest stages of vasculogenesis *in vivo*, as blood islands, endothelial cells and major vessel tubes fail to develop in appreciable numbers in embryos lacking either VEGF or VEGFR-2 (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Shalaby *et al.*, 1997; Shalaby *et al.*, 1995). On the contrary, VEGFR-1 seems to inhibit hemangioblast commitment, as the null embryos generate too many endothelial cells entering into the lumens of the abnormal vascular channels, leading to embryonic death at E8.5 (Fong *et al.*, 1999; Fong *et al.*, 1995).

Table 1. Summary of knockout studies of ligands and receptors regulating vasculogenesis and angiogenesis. See the text for references.

Gene	Null phenotype	Lethal at	Functions
VEGF	Defective EC development	E8-9 (-/-) E11-12 (+/-)	Induction of ECs, vasculogenesis, angiogenesis
PIGF	Impaired pathological angiogenesis	Survive	Recruitment of endothelial progenitor cells?
VEGF-B	Heart defects, impaired pathological angiogenesis	Survive	Modulation of VEGF activity (?)
VEGFR-1	Excess ECs, abnormal vessel structures	E8.5	Modulation of VEGFR-2 activity, pathological angiogenesis
VEGFR-2	Lack of endothelial and hematopoietic cells	E8.5-9.5	Hemangioblast migration, differentiation, proliferation
VEGFR-3	Defective vascular remodelling	E9.5-10.5	Blood vessel maturation, lymphatic vessel development and function
Ang-1	Impaired endocardial development and myocardial trabeculation, lack of pericytes	E12.5	Blood vessel stabilisation
Ang-2	Defective postnatal vascular remodelling, lymphatic drainage problems	<P14	Blood vessel destabilisation, lymphatic vessel development and function
Tie-1	Poor endothelial integrity, edema, and hemorrhage	E13.5-P0	EC-pericyte interactions
Tie-2	Heart trabeculation defects, decreased EC survinal and sprouting, hemorrhages	E9.5-10.5	EC-pericyte interactions
PDGF-B	Hemorrhage, loss of perivascular cells	P0	Pericyte recruitment
PDGF-Rβ	Hemorrhage, loss of perivascular cells	P0	Pericyte maturation, blood vessel stabilisation
Ephrin-B2	Defective vascular remodelling, organization and sprouting	E10.5	Establishment of arterial EC identity
EphB4	Defective vascular remodelling	E10.5	Establishment of venous EC identity

Angiogenesis

During the subsequent process of angiogenesis, ECs proliferate and sprout from pre-existing vascular network (Fig. 3). The primary capillary network is remodeled until a mature vasculature consisting of vessels of different diameters and functions is formed. This remodeling occurs by regression, sprouting, splitting or fusion of pre-existing vessels (Risau, 1997). During embryogenesis, most organs are vascularized by a combination of vasculogenesis and angiogenesis. However, some ectodermal tissues, such as the brain, become vascularized exclusively by angiogenic mechanisms.

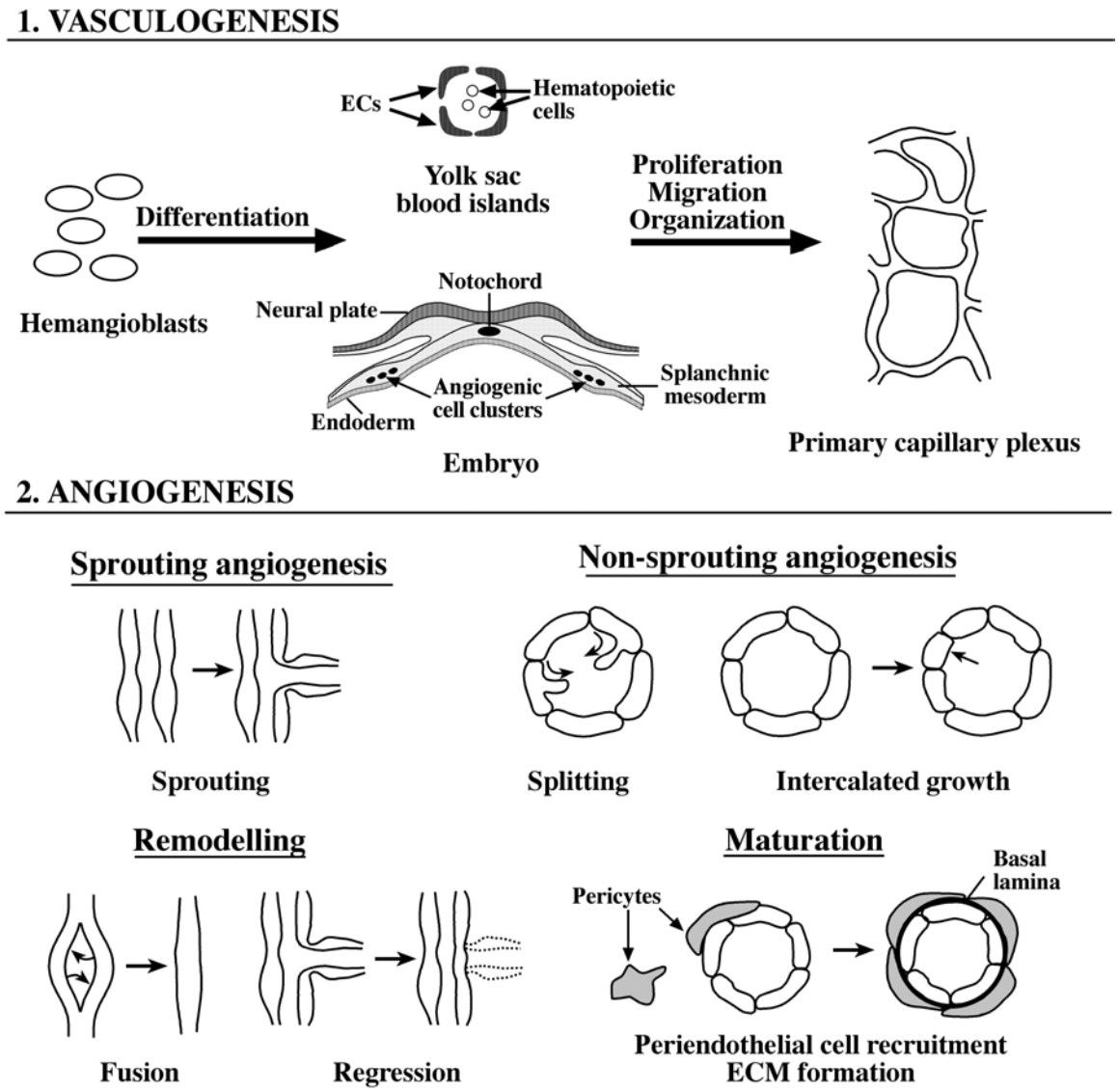

Figure 3. Embryonic blood vessel development. In vasculogenesis, endothelial cell precursors, angioblasts, differentiate in the yolk sac and in the embryo. Primordial vascular network is formed by fusion of the angioblasts. In angiogenesis, the primary capillary plexus is remodeled by regression and expansion of primitive vessels to form mature blood vessels, some of which are covered by periendothelial support cells. Adapted from Carmeliet, 2000.

Our understanding of the molecular players involved in angiogenesis has increased enormously during the last decade. In addition to its fundamental role in vasculogenesis, VEGF is the major regulator in angiogenesis. VEGF expression levels are tightly regulated during development, as even the loss of a single VEGF allele results in embryonic lethality at E11-12 (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). A variety of growth factors and cytokines including epidermal growth factor, basic fibroblast growth factor (bFGF), transforming growth factor-ß (TGF-ß), and keratinocyte growth factor-1 induce the transcription of VEGF mRNA (Ferrara, 1999). It is possible that these growth factors mediate the developmental regulation of VEGF. Also a lack of oxygen, hypoxia, is one of the major regulators of VEGF, inducing rapid and reversible VEGF expression by increasing its transcription and mRNA stabilization (Levy *et al.*, 1996; Levy *et al.*, 1995; Plate *et al.*, 1992; Shweiki *et al.*, 1992). Hypoxia-induced transcription of VEGF mRNA is mediated by hypoxia-inducible factor-1 (HIF-1) (Semenza, 1996). Hypoxia leads to stabilization of HIF-1 protein that is extremely labile in normoxic conditions, and it plays a role at least in some vascularization processes such as formation of the retina vasculature (Huang *et al.*, 1996; Stone *et al.*, 1995).

Recently, isoform-specific knock-outs of the VEGF gene have been generated. While mice expressing only the 164 aa isoform of VEGF are normal, those expressing only 120 or 188 aa isoforms display various defects (Carmeliet *et al.*, 1999b; Maes *et al.*, 2002; Stalmans *et al.*, 2002). Mice expressing only the 120 aa isoform of VEGF have defects in the heart, bone formation and retinal angiogenesis, whereas mice expressing only VEGF188 have impaired arteriolar development (Carmeliet *et al.*, 1999b; Maes *et al.*, 2002; Stalmans *et al.*, 2002). These results indicate that not all VEGF isoforms can completely replace the function of the others.

VEGF induces permeability, proliferation, migration, survival, sprouting and tube formation in cultured ECs (Ferrara, 1999). These responses are largely mediated by the VEGFR-2 receptor, although the binding affinity of VEGF for VEGFR-2 is lower than for VEGFR-1 (De Vries *et al.*, 1992; Terman *et al.*, 1992). VEGFR-1 has probably a modifying function, mostly by acting as a decoy receptor, as mutant mice expressing VEGFR-1 lacking its tyrosine kinase domain have normal vascular development (Hiratsuka *et al.*, 1998). Also VEGF-E, which does not bind to VEGFR-1, is able to carry out most of the functions that VEGF does *in vitro*, indicating that VEGFR-2 is the major receptor that conveys VEGF-induced signals in endothelial cells (Meyer *et al.*, 1999; Wise *et al.*, 1999). However, VEGFR-1 signaling is required for optimal monocyte and trophoblast function (Clauss *et al.*, 1996; Desai *et al.*, 1999).

VEGFR-2 works in conjunction with several adhesion receptors including vascular endothelial (VE)-cadherin and integrins during angiogenesis. Both the targeted deficiency and the cytosolic truncation of VE-cadherin in mice resulted in the same kind of vascular defects and embryonic lethality at E9.5 (Carmeliet *et al.*, 1999a). The defects were observed in VEGF-mediated angiogenesis and EC survival. VEGF in-

duces tyrosine phosphorylation of VE-cadherin (Esser *et al.*, 1998). Studies in cultured cells indicate that VE-cadherin plays a role in VEGF-mediated EC survival through VEGFR-2 (Carmeliet *et al.*, 1999a). The exact nature of VEGFR-2 and integrin co-signaling is unknown, but it has been speculated that integrins may enhance the clustering and dimerization of VEGFR-2 subunits in response to VEGF, thereby enhancing the VEGFR-2 signaling (Tallquist *et al.*, 1999).

In addition to VEGFR-1 and -2, VEGFR-3 is essential for normal development of the blood vasculature, as targeted inactivation of the VEGFR-3 gene in mice leads to defective remodeling of the primary vascular plexus, disturbed hematopoiesis and cardiovascular failure resulting in embryonic lethality at E9.5 (Dumont *et al.*, 1998; Hamada *et al.*, 2000). The exact roles of VEGF-C and VEGF-D during embryonic vascular development are still unknown due to lack of gene deletion studies. However, when VEGF-C was applied to the early chorioallantoic membrane (CAM), where lymphatics have not yet developed, it promoted angiogenesis, whereas in mature CAM it promoted lymphangiogenesis (Oh *et al.*, 1997). The angiogenic vs. lymphangiogenic role of VEGF-C may depend on the degree of proteolytic processing of its precursor and on the expression of its receptors. Recent data from several transgenic mouse studies suggest that VEGFR-3 and VEGF-C play a major role in lymphangiogenesis (see section: Development of the lymphatic vessels).

In contrast to the early defects in blood vessel formation in mice deficient of VEGF or its receptors, embryos lacking Tie receptors, Ang1 or Ang2 exhibit defects somewhat later in angiogenesis. Although the early stages of vascular development appear to occur rather normally, mice deficient of Tie1 die between E13.5 and birth due to compromised EC integrity leading to hemorrhages and abdominal edema (Puri *et al.*, 1995; Sato *et al.*, 1995). Embryos lacking Tie2 show an earlier lethal phenotype and die between E9.5 and E10.5 as a consequence of insufficient expansion and maintenance of the primary capillary plexus, failure of endothelial lining on the heart to develop properly and lack of capillary sprouts from the neuroectoderm (Dumont *et al.*, 1994; Sato *et al.*, 1995). Interestingly, double null mouse embryos for the Tie1 and Tie2 receptors have a more severe phenotype compared to single knockout phenotypes, suggesting that these receptors do biologically interact (Puri *et al.*, 1999). It has been suggested that Tie1 modulates angiopoietin signals via Tie2 (Maisonpierre *et al.*, 1997). Recently Tie1 and Tie2 receptor heterodimers have been shown to exist in cultured ECs, but Tie1 is not transphosphorylated on Tie2 receptor activation and the cellular function of heterodimerization remains to be determined (Marron *et al.*, 2000).

Ang1, the Tie2 agonistic ligand, is expressed most prominently in the heart myocardium at early stages of development (E9-11) (Maisonpierre *et al.*, 1997). Later, it becomes more widely distributed in the mesenchyme surrounding developing vessels. Mice deficient of Ang1 die at E12.5 due to similar angiogenic defects as seen in Tie2 knockout mice (Suri *et al.*, 1996). As in Tie2 deficient embryos, embryos lacking Ang1 have defects in trabecular formation of the heart and show a less complex vas-

cular network in general (Suri *et al.*, 1996). Also, as transgenic mice overexpressing Ang1 show hypervascularization, activation of the Tie2 signaling pathway is definitely involved in vascular network formation (Suri *et al.*, 1998). Ang2, the putative antagonistic ligand of Tie2, has a more punctate expression pattern than Ang1. During the early embryogenesis, Ang2 is expressed in the dorsal aorta and major aortic branches. Transgenic overexpression of Ang2 in the developing endothelium proved that Ang2 can act as a negative regulator of Tie2 as the mice had a phenotype reminiscent of that seen in embryos lacking either Ang1 or Tie2 leading to embryonic death at E9.5-10.5 (Hanahan, 1997; Maisonpierre *et al.*, 1997).

Vascular maturation

Stabilization of the vasculature occurs when periendothelial support cells such as SMCs and pericytes are recruited to the vessel wall and the surrounding ECM is reconstituted (Fig. 3). Large arteries, in which ECs are covered by a thick layer of SMCs, progressively branch into smaller vessels, terminating in precapillary arterioles. Arterioles are connected to capillaries, which are comprised almost entirely of ECs, with only occasional pericytes. Capillaries are connected to postcapillary venules that progressively associate into larger veins. Veins are fully enveloped by SMCs, although not to the same extent as arteries.

Vascular maturation is a critical step in blood vessel development, as in the absence of support cell coverage vessels undergo regression (Alon *et al.*, 1995). VEGF accelerates the formation of pericyte coverage, although the mechanism remains unknown (Benjamin *et al.*, 1998). Results from several studies indicate that Tie receptors are involved in the regulation of EC-pericyte/SMC interactions. A mutation in *TIE2* locus has been identified in some families with inherited venous malformations (Vikkula *et al.*, 1996). This mutation (R849W) increases the activity of TIE2 tyrosine kinase, leading to enlarged vascular channels with a relative lack of SMCs. The pericyte number is decreased also in the vasculature of Tie2 or Ang1 deficient embryos (Patan *et al.*, 1998; Suri *et al.*, 1996). Ultrastructural examination of vessels in mice lacking Ang1 showed that ECs are poorly associated with the underlying matrix and do not properly recruit and associate with periendothelial supporting cells (Suri *et al.*, 1996). Consistently, overexpression of Ang1 in the skin results in the formation of leakage-resistant vessels, indicating that Ang1 inhibits vascular permeability and stabilizes the existing vessels (Thurston *et al.*, 2000; Thurston *et al.*, 1999). In Tie1-deficient embryos, the pericyte numbers are increased, while platelet-derived growth factor (PDGF)-B deficient embryos, which show upregulated Tie1 expression, lack microvascular pericytes (Lindahl *et al.*, 1997; Patan, 1998). PDGF-B is the ligand for RTKs PDGFR-α and -ß (Heldin *et al.*, 1998). It is expressed in ECs and it promotes proliferation and migration of mesenchymal cells expressing the receptors. Targeted disruption of PDGF-B or PDGFR-ß genes in mice leads to hemorrhages and edema in late embryogenesis, demonstrating the vital role of periendothelial cells for the development of functional vasculature (Levéen *et al.*, 1994; Lindahl *et al.*, 1997; Soriano, 1994). Also the ECM between ECs and pericytes has an important function in vascular development as defects in the proper formation of the ECM lead to decreased pericyte

adhesion, migration and vessel stability (Beltramo *et al.*, 2002; Thyboll *et al.*, 2002). During subsequent arteriogenesis, some vessels become covered by a muscular coat, needed for viscoelastic and vasomotor functions.

Heterogeneity of the vascular endothelium

Vascular endothelium is not a uniform, semipermeable layer. Instead, it is a dynamic entity, capable of functional and morphological adaptations in response to environmental requirements. Based on current knowledge, the diversity of ECs is generated by both endogenous alterations and by exogenous stimuli from surrounding tissues.

On the basis of morphology, microvascular endothelium has been divided into different phenotypes: continuous, fenestrated and discontinuous (Pasqualini *et al.*, 2002). Capillaries in the skeletal muscle, heart, lung and brain are continuous. In these capillaries, the endothelial cytoplasm is continuous and there is no fusion of luminal and abluminal plasma membranes except at the cell junctions. Fenestrated and discontinuous capillaries have pores and gaps between ECs. Capillaries in many endocrine and exocrine glands, choroid plexus and intestinal villi have fenestrations. Functionally, fenestrated capillaries are more permeable than the continuous capillaries, which is consistent with their presence at sites of filtration, secretion and absorption. Capillaries in the liver, spleen and bone marrow have discontinuous endothelium with total or partial absence of basal lamina. Also the capillaries in different organs may be further specialized. This is the case for example in the brain, where capillaries form the blood-brain barrier. These capillaries have complex tight junctions between the ECs, which are primarily responsible for the barrier function needed to guarantee brain homeostasis. Because brain ECs are derived from external ECs by angiogenesis, the brain environment probably induces blood-brain barrier differentiation in the invading ECs. Indeed, astroglial cells are able to induce at least some characteristics of the blood-brain barrier phenotype in ECs (Rubin and Staddon, 1999). Also endocardium can be considered a distinct type of endothelium as it transdifferentiates to mesenchyme during formation of the cardial cushions (Sinning *et al.*, 1992).

Heterogeneity is evident also in large vessel endothelium, for example between arteries and veins. In addition to differences in structure and function, several genes are known to be differentially expressed between the developing arteries and veins. These include the Eph family transmembrane ligand ephrin-B2, which is expressed only in the arteries and its receptor, EphB4, which is expressed only in veins (Wang *et al.*, 1998). In fact, the differential expression pattern between ephrin-B2 and EphB4 is seen in the developing vessels already before the onset of circulation, indicating that arteries and veins are genetically determined. Embryos lacking ephrin-B2 or EphB4 display severe vascular defects including aberrant vessel remodeling and heart development, leading to embryonic death (Adams *et al.*, 1999; Gerety *et al.*, 1999; Wang *et al.*, 1998). Defects are seen in both arteries and veins, indicating that bidirectional signaling needed for normal vascular development occurs between ephrin-B2 and EphB4 (Gerety *et al.*, 1999; Wang *et al.*, 1998).

Little is known about the upstream factors responsible for defining the differences between arteries and veins. The activin receptor like kinase-1 (*ACVRL1*) participates in the determination of arteries and veins, as a loss of function mutation in *ACVRL1* causes arteriovenous malformations (Johnson *et al.*, 1996; Urness *et al.*, 2000). Mice lacking *Acvrl1* fail to express ephrin-B2 in their arterial vessels, suggesting a possible role for TGF-ß signaling during this process (Urness *et al.*, 2000). In addition, Notch and gridlock pathways regulate the determination of arterial or venous endothelial cell fate (Lawson *et al.*, 2001; Zhong *et al.*, 2001). Results from zebrafish studies indicate that angioblasts of the lateral mesoderm are already restricted to an arterial or venous lineage (Zhong *et al.*, 2001). Lack of either Notch or gridlock (grl) expression leads to a failure to induce ephrin-B2 and to enhanced expression of venous markers, indicating that these signaling pathways are needed for repression of the venous fate in order for the arterial fate to emerge. Recent data suggest that sonic hedgehog (Shh) acts upstream of VEGF and Notch, and participates in the induction of arterial differentiation (Lawson *et al.*, 2002). Interestingly, Shh induces the expression of VEGF, Ang1 and Ang2 (Pola *et al.*, 2001). However, the major role of Shh in arterial differentiation is to induce the expression of VEGF. At present, it is unclear whether VEGF is responsible for the initial specification of arterial progenitors or their subsequent differentiation. It has been speculated that continuous exposure to VEGF would induce the Notch signaling pathway, thereby increasing the level of grl in arterial progenitors (Lawson *et al.*, 2002).

Angiogenesis in the adult

In addition to its vital role during embryogenesis, angiogenesis is required after birth for the growth of several organs. Partial inhibition of VEGF by a soluble extracellular region of VEGFR-1 resulted in increased mortality and impaired growth plate morphogenesis and endochondral bone formation in the early postnatal period (Gerber *et al.*, 1999). ECs of mature, quiescent vessels in the adult have a very low proliferation rate, with estimated turnover times measured in months or even years. However, new vessel growth occurs in some physiological conditions, such as in the female reproductive cycle and endocrine function of the ovarian corpus luteum (Ferrara *et al.*, 1998). The expression of VEGFRs and Tie receptors is decreased in quiescent adult endothelium in comparison to embryonic endothelium, and increased during periods of angiogenesis (Korhonen *et al.*, 1992; Kremer *et al.*, 1997; Otani *et al.*, 1999; Wong *et al.*, 1997). New vessels in the adult arise mainly through angiogenesis, although recent studies indicate that vasculogenesis, incorporation of endothelial cell precursors to the developing vessels, may also occur (Asahara *et al.*, 1999; Asahara *et al.*, 1997; Takahashi *et al.*, 1999).

Angiogenesis is initiated by vasodilation and by an increase in vascular permeability in response to angiogenic growth factors. VEGF has a central importance in this process. Neovascularization of female reproductive system is largely mediated by hormonally induced expression of VEGF. In vessels undergoing active remodeling, the stabilizing function of Ang1 is overcome by an excess of Ang2 expression (Maisonpierre *et al.*, 1997). Ang2 expression makes endothe-

lial cells prone to apoptosis. However, if VEGF is expressed simultaneously, endothelial cells proliferate and form new vessels (Holash *et al.*, 1999). At sites of active vessel growth e.g. in ovary and in tumors, Ang2 expression precedes that of VEGF. It has been speculated that Ang2 is able to trigger the detachment of pericytes, making ECs more vulnerable to angiogenic stimuli. In addition, VEGF induces the expression of EC surface integrins, which directly regulate EC interactions with the ECM. Integrins are important for VEGF-driven angiogenesis, as blocking antibodies for EC surface integrins inhibited neovascularization (Senger *et al.*, 1997). Once ECs are activated, they start to produce proteases that degrade the basement membrane and liberate growth factors sequestered within the ECM. These growth factors further stimulate the ECs to migrate, proliferate and to form new vessels. Finally the expression of PDGF-B gets upregulated in ECs to attract periendothelial support cells and to stabilize the vessels.

In addition to physiological angiogenesis, blood vessel growth is a major feature in many pathological conditions such as wound healing, ischemic heart diseases, diabetic retinopathy, tumor growth and metastasis. Angiogenesis is beneficial in wound healing and for the development of collateral vessels in ischemic diseases. Tissue ischemia induces the expression of several angiogenic growth factors and their receptors, thereby promoting angiogenesis (Semenza, 2001). In some pathological conditions, however, increased angiogenesis is detrimental. These include diabetic retinopathy, rheumatoid arthritis and psoriasis. Although several mechanisms regulating pathological and physiological blood vessel growth are alike, some molecules only minimally involved in embryonic vascular development do affect pathological angiogenesis (Carmeliet, 2000). One pair of such molecules is PlGF and VEGFR-1. Systemic application of PlGF, which mediates its effect only via VEGFR-1, enhances angiogenesis in ischemic adult tissues (Luttun *et al.*, 2002). VEGF-B is another example, as VEGF-B deficient mice are overall healthy and fertile having only very mild heart phenotypes (Aase *et al.*, 2001; Bellomo *et al.*, 2000). However, after coronary occlusion or myocardial ischemia, mice lacking VEGF-B show impaired recovery (Bellomo *et al.*, 2000). Another difference between physiological and pathological angiogenesis is that the latter is often induced by inflammation. For example in healing wounds, inflammatory cells including macrophages, monocytes and leukocytes secrete cytokines stimulating the proliferation and migration of endothelial cells and support cells (Frank *et al.*, 1995).

In order to grow beyond a few cubic millimeters in size, tumors must promote new blood vessel growth (Folkman *et al.*, 1996). Inhibitors of angiogenesis maintain a physiological balance with stimulators of endothelial proliferation. The switch from pre-vascular to angiogenic state in tumors involves the disruption of this balance. In contrast to regular morphology and blood flow in normal vasculature, tumor vessels are disorganized and leaky and the tumor blood flow is chaotic and variable (Baish and Jain, 2000; Hashizume *et al.*, 2000; Morikawa *et al.*, 2002). In several tumors, increased vascularization has been directly correlated with poor prognosis. Angiogenesis is also required for the estab-

lishment and growth of metastases at secondary sites. In fact, a direct correlation between tumor vascularity and metastatic potential has been shown in a variety of tumor types, including non-small cell lung carcinoma, breast carcinoma and gliomas (Leon *et al.*, 1996; Marcchiarini *et al.*, 1992; Weidner *et al.*, 1991).

The expression of many angiogenic growth factors and their receptors is upregulated in tumors (reviewed in Ferrara and Alitalo, 1999; Saaristo *et al.*, 2000). Overexpression of VEGF is a common feature of tumors, and specific inhibition of its function by a blocking monoclonal antibody inhibits tumor vascularization and growth (Kim *et al.*, 1993). Interruption of VEGFR-2 or Tie2 signaling pathways via delivery of dominant-negative VEGFR-2 receptor or soluble extracellular domains of VEGFR-1 or Tie2 inhibits tumor growth in mice, indicating importance of these receptors for tumor angiogenesis (Goldman *et al.*, 1998; Lin *et al.*, 1997; Lin *et al.*, 1998a; Millauer *et al.*, 1996; Millauer *et al.*, 1994; Siemeister *et al.*, 1999). Also VEGFR-3 is expressed in capillary vessels in tumor angiogenesis (Partanen *et al.*, 1999a; Valtola *et al.*, 1999). Inactivation of VEGFR-3 by neutralizing antibodies supresses tumor growth by destabilizing tumor-associated angiogenic vessels, without affecting the established blood and lymphatic vasculature (Kubo *et al.*, 2000).

1.2.2 The lymphatic system

Lymphatic system collects extravasated fluid and lymphocytes from the tissues into blind-ended lymphatic capillaries, and transports them back to the blood circulation (Fig. 3). Unlike the blood vasculature, which forms a circulatory loop, the lymphatic system forms a one-way transit system. The structure of lymphatic capillaries is also different from blood capillaries, reflecting the difference in their function (Fig. 3). Lymphatic capillaries consist of an extremely permeable, thin EC layer that is often devoid of surrounding support cells and basement membrane. Anchoring filaments connect lymphatic ECs to the ECM and hold the vessels open when the pressure rises in the interstitial tissue (Swartz, 2001). From these small lymphatic vessels, lymph is transferred to progressively larger collecting lymphatics, consisting of endothelial, muscular and adventitial layers, and ultimately into the venous circulation via the thoracic duct. In addition to the lymphatic vessels, the lymphatic system also contains several lymphoid organs (spleen, lymph nodes, tonsils and thymus) that are essential in immune responses. Furthermore, lymphatic vessels participate in absorption of lipids from the gut (Witte *et al.*, 2001).

Development of the lymphatic vessels

The development of the lymphatic vascular system begins in the fifth week of embryonic life in humans. When the embryo grows, lymphatic vessels are needed for the regulation of the interstitial tissue pressure. According to present data, lymphatic vessels originate from lymph sacs, which are formed by endothelial sprouting from the veins (Dumont *et al.*, 1998; Wigle *et al.*, 1999) (Fig. 4). It is also possible that lymphangioblasts exist and participate in lymphatic vessel development in mammals. In avians primitive lymphangioblasts, which differentiate from the mesenchyme *in situ* to lymphatic endothelial cells, have been identified and these cells can be re-

cruited by the developing lymphatic vessels (Schneider *et al.*, 1999).

Recently, various markers specific for the lymphatic endothelium have been characterized, enabling the study of lymphatic vascular development. The first indication of lymphangiogenesis is seen around E9.5 in mouse, when the expression of a homeobox transcription factor Prox-1 begins. Prox-1 has an important role in the formation of the lymph sacs by endothelial sprouting from the veins (Wigle *et al.*, 2002; Wigle and Oliver, 1999). Mice deficient of Prox-1 do not develop any lymphatic vessels, which results in severe edema at midgestation and death around E14.5 (Wigle and Oliver, 1999). Further support for the importance of Prox-1 in defining lymphatic EC characteristics came from studies of cultured cells. Recently techniques allowing the isolation of primary human blood and lymphatic ECs have been developed (Kriehuber *et al.*, 2001; Mäkinen *et al.*, 2001b). The gene expression profiles in these two cell populations have been analyzed and a number of genes were found to be differentially expressed (Petrova *et al.*, 2002). Interestingly, ectopic overexpression of Prox-1 in blood ECs induces the expression of about one third of lymphatic EC-specific genes, indicating that Prox-1 is an important regulator of lymphatic differentiation (Petrova *et al.*, 2002). After the initial budding from embryonic veins, lymphatic ECs start to express additional lymphatic endothelial markers, and the simultaneous expression of LYVE-1, Prox-1, VEGFR-3 and SLC in ECs indicates commitment to the lymphatic EC lineage (Wigle *et al.*, 2002).

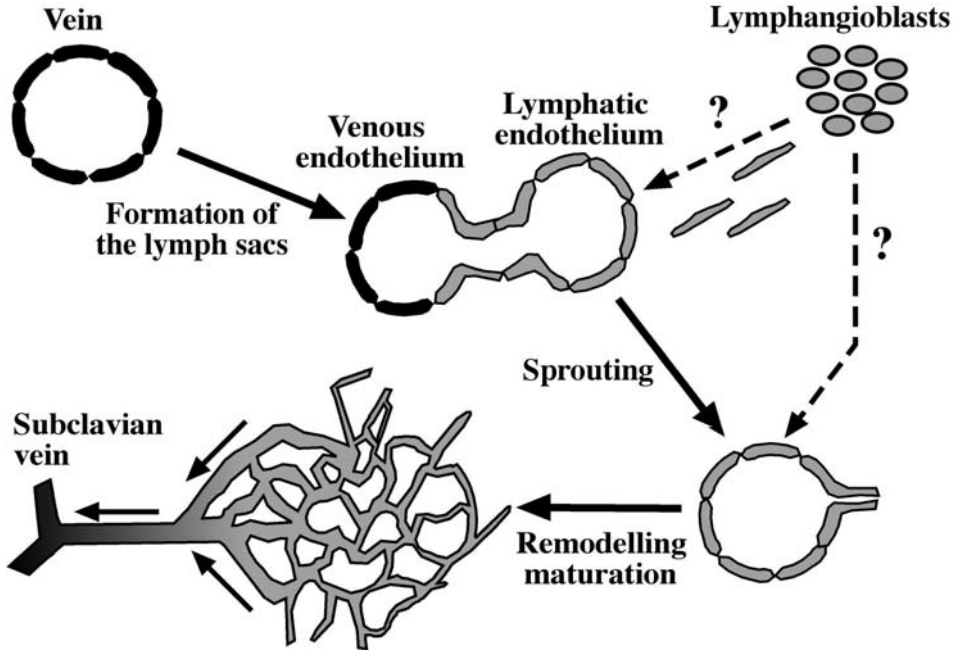

Figure 4. Embryonic lymphatic vascular development. Florence Sabin proposed already in 1902 that primitive lymph sacs form by sprouting from embryonic veins. Peripheral lymphatic system then spreads from these lymph sacs. Also lymphatic precursor cells (lymphangioblasts) differentiating *in situ* from the mesenchyme to form lymphatic endothelial cells may exist and participate in lymphatic vessel formation. Adapted from Saaristo, 2002b.

The expression of VEGFR-3 is maintained in budding lymphatic ECs, while its expression becomes weaker in the blood vasculature. VEGFR-3 expression is largely restricted to the lymphatic endothelium around E14 in mouse development (Kaipainen *et al.*, 1995). A paracrine expression pattern is seen between VEGF-C and VEGFR-3 at sites where the first lymphatic sprouts occur, suggesting that VEGF-C and VEGFR-3 play a major role in the development of lymphatic vessels (Kukk *et al.*, 1996). The expression pattern of the other ligand for VEGFR-3, VEGF-D, has not been studied in detail but its mRNA has been observed in the developing melanocytes and fibroblasts, lung mesenchyme and in the adult vascular wall (Achen *et al.*, 2001). Whether the function of VEGF-C and/or VEGF-D is critical for the lymphatic vessel development remains to be ascertained as the phenotypes of VEGF-C and VEGF-D null mice have not been published yet.

In addition to VEGFR-3, VEGFR-1 and VEGFR-2 are also expressed in lymphatic endothelium (Mäkinen *et al.*, 2001b; Saaristo *et al.*, 2002a). However, VEGFR-3 expression pattern differs from the two other VEGFRs, as it is relatively specific for lymphatic endothelial cells. Few exceptions to the lymphatic VEGFR-3 expression pattern are known, as it is expressed in some fenestrated blood endothelial cells and angiogenic blood vessels in breast tumors (Partanen *et al.*, 2000; Valtola *et al.*, 1999). The importance of VEGFR-3 for lymphatic vessel development is illustrated by the results from several transgenic mouse studies. Mice expressing soluble ligand binding part of the VEGFR-3 in the skin keratinocytes have normal blood vasculature, but lack dermal lymphatics (Mäkinen *et al.*, 2001a). In contrast, overexpression of VEGF-C or VEGF-D resulted in hyperplasia of the superficial lymphatic vessels, again without any blood vascular effects (Jeltsch *et al.*, 1997; Veikkola *et al.*, 2001). Interestingly, overexpression of a mutant form of VEGF-C (VEGF-C156S) that is a specific ligand for VEGFR-3 also leads to lymphatic dilation in transgenic mice, indicating that signaling via VEGFR-3 alone is sufficient for lymphangiogenesis *in vivo* (Veikkola *et al.*, 2001).

There are differences in the expression patterns of VEGFR-2 and VEGFR-3 in different types of lymphatic vessels. VEGFR-3 is highly expressed by the initial lymphatic capillaries while VEGFR-2 expression is highest in the collecting lymphatic vessels in the adults (Saaristo *et al.*, 2002a). To date, there is no published data on lymphatic VEGFR-1 expression *in vivo*. Like VEGF, VEGF-C increases vascular permeability and stimulates EC migration and proliferation but at higher concentrations than VEGF (Mäkinen *et al.*, 2001b). VEGF-C156S and VEGF-D, on the other hand, have been reported to be inactive in the permeability assay (Achen *et al.*, 1998; Joukov *et al.*, 1998). The lymphatic effects induced by VEGF-C and VEGF-C156S overexpression *in vivo* are different. While VEGF-C156S overexpression results in lymphatic hyperplasia, VEGF-C induces intensive lymphatic sprouting, suggesting that VEGFR-2 activation may be required for efficient induction of lymphatic sprouting (Saaristo *et al.*, 2002a).

Recent findings indicate that Ang2 mediates lymphangiogenic signals although it is not required for the initial formation of lymphatics (Gale *et al.*, 2002). Ang2 deficient mice die by two weeks of age due to lymphatic defects. The large mesenteric lymphatic vessels are poorly associated with SMCs, and the smaller lymphatic vessels in the gut and in the skin are disorganized and irregular. Mice having Ang2 gene replaced by Ang1 have no lymphatic defects, suggesting that Ang2 functions as an agonist in lymphatic ECs. Both Tie receptors are expressed at least in some lymphatic ECs in human fetal heart (Partanen *et al.*, 1999b). Also, Tie1 is expressed in the lymphatic vessels in K14-VEGF-C transgenic mice (Jeltsch *et al.*, 1997). However, no lymphatic defects have been characterized in Tie deficient mice, probably due to embryonic death caused by vascular defects before the lymphatic phenotype would appear.

In addition to Ang2 knockouts, mice with a targeted disruption of Net, a member of Ets transcription factors, or integrin α9, die neonatally because of lymph drainage problems (Ayadi *et al.*, 2001; Huang *et al.*, 2000). At present, it is not known whether Net and integrin α9 regulate lymphatic vessel function via VEGFRs or Tie receptors or via alternative unidentified pathways.

Lymphangiogenesis in the adult

Lymphangiogenesis appears to follow but lag behind angiogenesis in adult tissues. In healing skin wounds, VEGFR-3 positive lymphatic vessels sprout from pre-existing lymphatics and grow into the granulation tissue (Paavonen *et al.*, 2000). The reason for the delay in lymphatic vessel growth in comparison to blood vessel growth may be the fact that blood vascular endothelial cells produce VEGF-C, which is needed to attract lymphatic vessels (Kriehuber *et al.*, 2001; Mäkinen *et al.*, 2001b).

Abnormal lymphatic vessel development and function are associated with human lymphedema. Lymphedema is characterized by disfiguring and disabling swelling of the limbs due to defective lymphatic drainage. Several missense mutations within the *VEGFR3* region have been linked to human primary lymphedema (Irrthum *et al.*, 2000; Karkkainen *et al.*, 2000). Other genetic loci are linked to other lymphedema syndromes. Lymphedema-distichiasis, for example, has been linked to mutations in the *FOXC2* gene (Fang *et al.*, 2000). FOXC2 is a transcription factor but so far it is not known how it regulates lymphatic vessel growth or function. Mice deficient of *Foxc2* did not give an answer to that question, as they die during embryogenesis or perinatally due to vascular defects and do not have lymphedema (IIda *et al.*, 1997; Winnier *et al.*, 1997). Although several mutations have been linked to lymphedema, the most common form of lymphedema is not genetic but results from filarial worm infection causing the obstruction of lymphatics.

The lymphatic system serves as the primary pathway for metastatic spread of tumor cells to regional lymph nodes. Recent studies in mice have indicated that overexpression of VEGF-C or VEGF-D in tumors leads to extensive lymphangiogenesis in/around tumors and it is associated with increased frequency of metastases (Karpanen *et al.*, 2001; Mandriota *et al.*, 2001; Skobe *et al.*, 2001; Stacker *et al.*, 2001). However, it is still unclear whether VEGF-C or VEGF-D

induce lymphangiogenesis in human tumors and whether tumor lymphangiogenesis plays an important role in tumor growth and metastasis in humans. A correlation has been found between VEGF-C expression and formation of metastasis in several human cancers, suggesting that this may be the case (Akagi *et al.*, 2000; Bunone *et al.*, 1999; Tsurusaki *et al.*, 1999).

2 REGULATION OF ENDOTHELIAL CELL-SPECIFIC EXPRESSION OF GENES

2.1 Characterization of regulatory elements of endothelial specific genes

Gene expression can be controlled at several stages, such as transcription, hnRNA processing, translation and post-translational modification. The promoters of eukaryotic protein-encoding genes utilize a large combinatorial array of DNA elements and protein transactivators as receivers of physiological cues to direct spatial and temporal programs of gene expression. Transcription is controlled at the stage of initiation, as a result of modulation of transcription factor expression or post-translational modifications. Transcription factors can activate RNA polymerase II driven transcription via a promoter or an enhancer. The promoter determines the site of transcriptional initiation, which is carried out when transcription factors bind to the DNA sequence close to and upstream of the start site. The enhancer stimulates promoters in a temporally regulated or tissue-specific manner, when transcription factors bind to specific sequences distal to and either upstream or downstream from the start site (Blackwood and Kadonaga, 1998). In contrast to the promoter, enhancer functions in an orientation independent manner. There are several possibilities how enhancers regulate gene expression. Enhancers may stimulate promoters by relieving chromatin-mediated repression, a process that may involve histone acetylation. They may be responsible for locating the template at a particular place within the cell. Enhancers can also function in stimulating DNA replication. Cell type specific expression can be regulated also via silencers, which inhibit the transcription of certain genes in other than target tissues.

Although significant progress has been made in determining the role of endothelial cell surface molecules in the process of vascular development, little is known about the mechanisms of differentiation and maturation of ECs. However, during the last few years, the *cis*-acting DNA elements and *trans*-acting protein factors that regulate transcription of several EC-specific genes have been identified and partially characterized. The results from these studies are summarized below.

2.1.1 The *Tie* genes

Both *Tie1* and *Tie2* are expressed already in the embryonic angioblasts and continue to be expressed mainly in the vascular endothelium throughout embryonic development. However, Tie receptors are also expressed in some hematopoietic stem cells and B-cells as well as in leukemic cell lines (Armstrong *et al.*, 1993; Hashiyama *et al.*, 1996; Iwama *et al.*, 1993; Kukk *et al.*, 1997; Yano *et al.*, 1997). In comparison to the

embryonic vasculature, Tie1 and Tie2 expression is downregulated in quiescent adult endothelia, although receptors can be detected in most adult endothelia as well.

The genomic regulatory regions of *Tie1* and *Tie2* necessary for targeting gene expression specifically to the endothelium have been identified (Korhonen *et al.*, 1995; Schlaeger *et al.*, 1997) (Fig. 5). Neither *Tie1* nor *Tie2* promoters contain TATA or CAAT boxes, which are often present in promoter elements. The 5´ regions of both mouse and human *Tie1* genes have been partially characterized and the promoter fragments sufficient to target expression of heterologous genes specifically to ECs *in vivo* have been determined (Korhonen *et al.*, 1995). Analysis of transgenic mice expressing a *LacZ* reporter gene driven either by a 0.8 kbp mouse *Tie1* promoter fragment or by a 5 kbp human *TIE1* promoter fragment showed activity in endothelia throughout development and in adulthood (Korhonen *et al.*, 1995). Comparison of mouse and human *Tie1* promoter sequences revealed several conserved putative binding sites for transcription factors conforming to Ets and AP-2 families as well as a GT-rich region and an octameric element, indicating that these DNA elements might have a regulatory role.

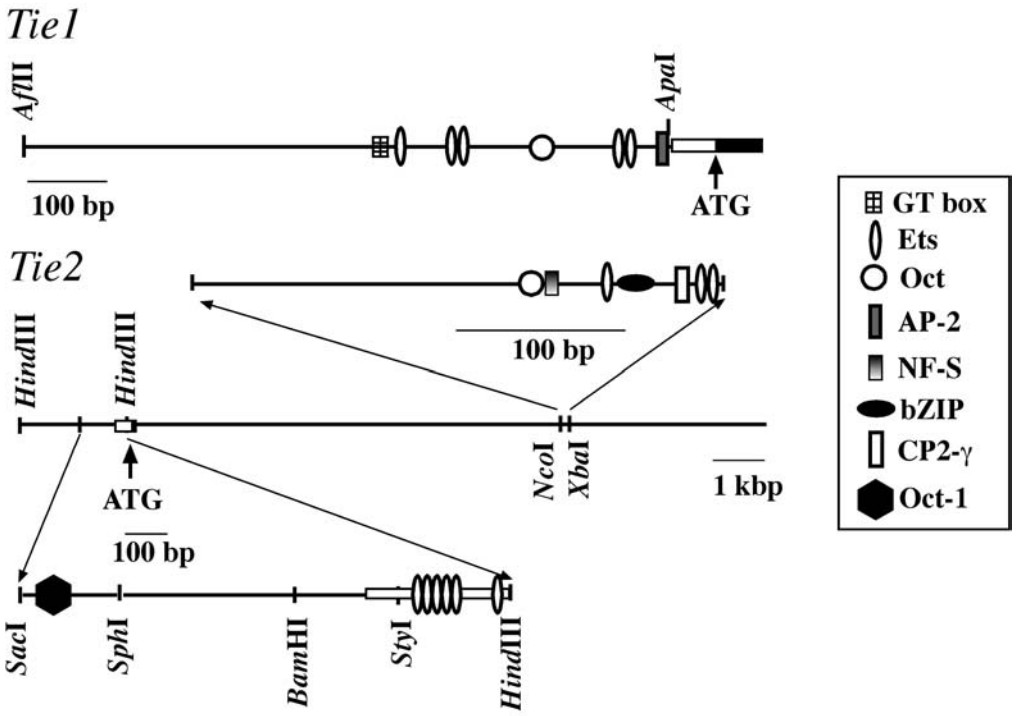

Figure 5. Regulatory elements of *Tie1* and *Tie2* genes. In *Tie1*, 0.8 kbp proximal promoter is needed to target gene expression specifically to endothelial cells. In *Tie2*, the endothelial regulatory elements are distributed in both the proximal promoter and in the large first intron (particularly in the 303 bp *Nco*I-*Xba*I fragment). The regulatory regions share several DNA motifs including putative binding sites for the Ets transcription factors as well as an octamer factor binding site. In addition, a putative transcription factor binding site for AP-2 is found in the *Tie1* promoter and sites for Oct-1, NF-S, bZIP and CP2–γ are found in the *Tie2* promoter/intronic enhancer. White boxes indicate the non-coding portions of the first exons and black boxes indicate the coding sequences; the arrows with ATG indicate the sites of the translational initiation codons. Adapted from Jones *et al*., 2001.

Similar transgenic mouse experiments with the *LacZ* reporter have been carried out with several 5´ flanking fragments of mouse *Tie2*. In the case of *Tie2*, a 1.2 kbp promoter fragment was sufficient to drive reporter gene expression in endothelia in the early embryonic stages (Schlaeger *et al.*, 1995). The EC-specific activity of *Tie2* promoter has been located to a 223 bp 5´ untranslated region, as deletion of that region abolished the EC-specific activity *in vivo* (Schlaeger *et al.*, 1995). Further analysis of this critical *Tie2* promoter region revealed the importance of a consensus octamer binding element. Based on results from EMSA experiments, this octamer forms a complex with an ubiquitous factor Oct1 and an EC-specific cofactor (Fadel *et al.*, 1999). Moderate reporter gene activity driven by the 2.1 kbp *Tie2* promoter was observed also in the brain and kidney of adult mice, whereas the regulatory elements present within the first intron of *Tie2* were needed for more extensive expression in both embryonic and adult endothelium (Evans *et al.*, 2000; Schlaeger *et al.*, 1997) (Fig. 5). An internal 1.7 kbp intronic fragment was identified as an autonomous EC-specific enhancer, as it could activate heterologous *tk* promoter in both orientations in the endothelia of transgenic mice (Schlaeger *et al.*, 1997). The core enhancer activity in the *Tie2* intronic sequence was found to be located within a 303 bp fragment. Sequence analysis of this fragment revealed the presence of several putative binding sites for general and tissue-specific transcription factors (Fig. 5). Mutational analysis of these sites indicated that the octameric palindrome and the putative NF-S site regulate the integration site dependency of the reporter construct, whereas mutations made either to the single Ets binding site (EBS) or to the region containing putative sites for bZIP, CP2-gamma and two EBSs render the enhancer completely inactive (Schlaeger *et al.*, 1997).

Both Tie1 and Tie2 protein levels are increased by hypoxia (McCarthy *et al.*, 1998; William *et al.*, 2000). Hypoxic activation of Tie2 is mediated most likely at least partly by the hypoxia-inducible factor-1α (HIF1α)-related transcription factor HIF-2/EPAS1/HRF. HIF-2 is expressed in the endothelium and its cotransfection strongly induces the *Tie2* promoter/enhancer activity (Schlaeger *et al.*, 1997; Tian *et al.*, 1997).

2.1.2 The *VEGFR1* and *VEGFR2* genes

The expression patterns of *VEGFR1* and *VEGFR2* are quite similar to each other as both receptors are expressed primarily in ECs, their expression is downregulated in adult endothelia and upregulated during tumor angiogenesis. However, some differences have been detected for example in the fetal human heart, as only *VEGFR1* was expressed in coronary endothelium (Kaipainen *et al.*, 1993; Partanen *et al.*, 1999b). There are also differences in the non-endothelial expression pattern of *VEGFR*s, as *VEGFR1* is expressed in macrophages, monocytes, trophoblasts and renal mesanglial cells, whereas *VEGFR2* is expressed in hematopoietic stem cells, megakaryocytes, platelets and retinal progenitor cells (Barleon *et al.*, 1996; Charnock-Jones *et al.*, 1994; Clauss *et al.*, 1996; Katoh *et al.*, 1995; Takahashi *et al.*, 1995; Yang and Cepko, 1996; Ziegler *et al.*, 1999).

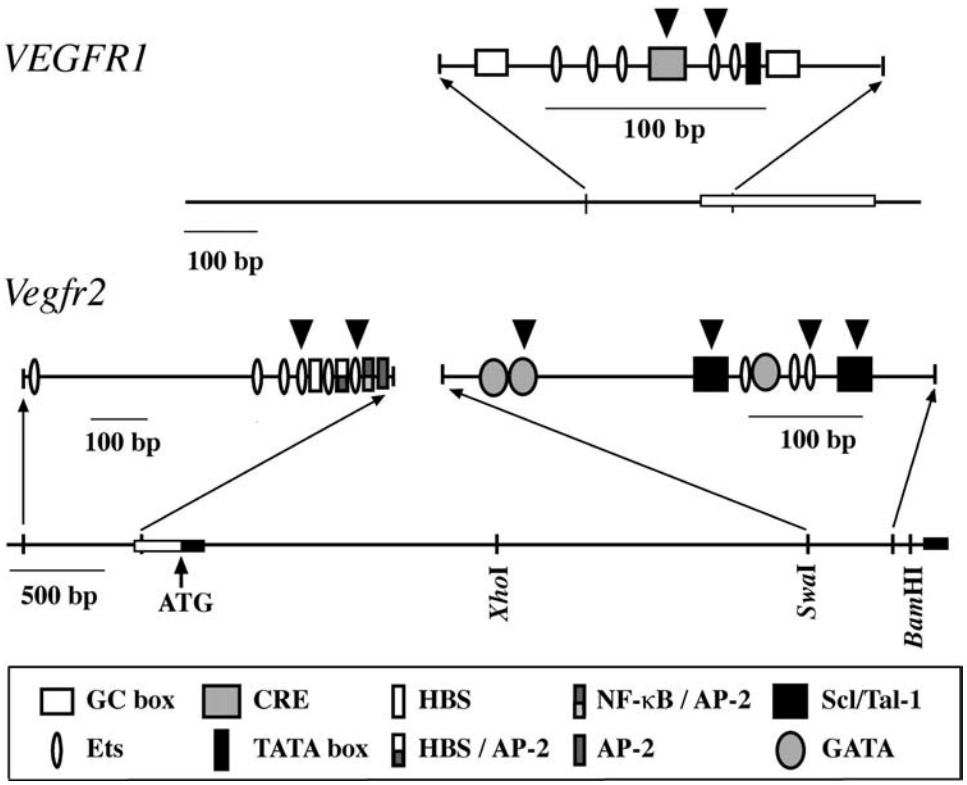

Figure 6. Schematic structure of *VEGFR1* and *Vegfr2* regulatory regions. In *VEGFR1* promoter the critical region for EC-specific expression contains a TATA box, one CRE motif, two GC boxes and multiple Ets binding sites. The promoter region of *Vegfr2* contains multiple binding sites for transcription factors conforming to Ets, AP-2, NF-κB and GATA families as well as two HIF binding sites (HBSs). Transcription factor binding sites including several GATA, Scl/Tal-1 and Ets sites present within the EC-specific enhancer, which is located immediately upstream of the second exon are indicated. The critical transcription factor binding sites are pointed out by arrowheads, see text for details.

The 5′ flanking regions of both mouse and human *VEGFR1* have been cloned and partially characterized. The 2.2 kbp mouse *Vegfr1* promoter fragment directs endothelial specific reporter gene expression in embryoid bodies (Quinn *et al.*, 2000). EC-restricted reporter gene expression is detected also by the 1 kbp human *VEGFR1* promoter fragment after adenoviral cell delivery *ex vivo* (Nicklin *et al.*, 2001). Furthermore, this promoter fragment is sufficient for directing widespread vascular expression in the embryo and in the endothelium of most organs in adult mice (Minami *et al.*, 2002). *VEGFR1* promoter has a TATA box, and an approximately 200 bp sequence upstream of exon 1 was found to confer endothelial cell type specific reporter gene expression in cultured cells (Fig. 6) (Ikeda *et al.*, 1996; Morishita *et al.*, 1995). This region contains several motifs for transcription factors including one cAMP response (CRE)/ATF element, several EBS and two GC boxes (Ikeda, 1996 *et al.*; Morishita *et al.*, 1995). Mutation of the CRE or single EBS just downstream of CRE decreased the basal activity by 90% (Wakiya *et al.*, 1996). Of the Ets factors, Ets1, Ets2 and Erg are able to stimulate *VEGFR1* promoter activity *in vitro* (Dube *et al.*, 1999; Wakiya *et al.*, 1996). Both the CRE motif and EBS need to be intact for

efficient Ets transactivation, indicating cooperative relations between these motifs in the *VEGFR1* promoter (Wakiya *et al.*, 1996). Interestingly, positive correlation was observed between *ETS1* and *VEGFR1*, but not with *VEGFR2* expression in the endothelial cells of human gliomas (Valter *et al.*, 1999). Targeted inactivation of JunB, a member of the AP-1 transcription factor family, leads to defective yolk sac vascularization in mouse embryos due to reduced *Vegfr1* expression (Schorpp-Kistner *et al.*, 1999). The Jun proteins form either homo- or heterodimers with members of the Fos and ATF protein families (Vogt and Bos, 1990). However, when co-transfected with other members of the AP-1 transcription factor complex, c-Fos and c-Jun do not transactivate the *VEGFR1* promoter fragment containing the CRE motif (Wakiya *et al.*, 1996). The first intron of the *VEGFR1* negatively regulated gene expression, possibly due to transcriptional arrest (Morishita *et al.*, 1995).

Also the regulatory elements of the mouse and human *VEGFR2* have been identified (Kappel *et al.*, 1999; Patterson *et al.*, 1995; Ronicke *et al.*, 1996). A 4 kbp fragment of *VEGFR2* promoter directed high level luciferase activity in endothelial cells but not in other cell types (Patterson *et al.*, 1995). Similarly, the 2.2 kbp *Vegfr2* promoter showed strong endothelium specific activity in transfected cells (Ronicke *et al.*, 1996). The *VEGFR2* promoters contain no TATA consensus sequence but they are GC rich and contain several Sp1 elements. Single transcription start sites were identified in human and mouse promoters, located 303 bp and 299 bp 5' of the translational start, respectively. Results from *in vivo* footprinting experiments suggest that Sp1 participates in cell type specific *VEGFR2* expression as Sp1 sites are occupied only in endothelial cells (Patterson *et al.*, 1997). Transient transfection assays with several *VEGFR2* promoter-reporter constructs revealed additional positive regulatory elements with conserved potential binding sites for AP-2, NF-κB and E-Box proteins (Patterson *et al.*, 1995). Transactivation studies indicate that Ets1 and Ets2 can activate the 0.9 kbp *Vegfr2* promoter fragment (Kappel *et al.*, 2000). Ets1 transactivates *Vegfr2* promoter via two of the six putative EBSs (Kappel *et al.*, 2000). One of these two EBSs is required to target high level reporter gene expression to ECs *in vivo* (Kappel *et al.*, 2000). In addition to EBSs, the *Vegfr2* promoter region needed for adequate promoter activity in cultured cells has several AP-2 sites and one potential NF-κB site (Fig. 6) (Ronicke *et al.*, 1996).

Despite the activity in cultured ECs, *Vegfr2* promoter sequences are not sufficient to confer reproducible EC-specific reporter gene expression *in vivo* (Kappel *et al.*, 1999). However, in combination with a 2.3 kbp fragment from the first intron of the *Vegfr2* gene, the 0.9 kbp *Vegfr2* promoter could drive reporter gene expression specifically in ECs in transgenic mice throughout the development (Kappel *et al.*, 1999). The proximal *Vegfr2* promoter mainly contributes to strong and position-independent reporter gene expression, while the EC-specificity of *Vegfr2* is mediated by the intronic enhancer. A 430 bp sequence of the intronic enhancer is sufficient for targeting endothelial-specific reporter gene expression *in vivo*. Several potential binding sites for the transcription factors of Scl/Tal-1 (Stem cell leukemia/T-cell acute leukemia),

GATA and Ets families are located in the minimal enhancer (Fig. 6). Scl/Tal and GATA transcription factors have been proposed to play a role in vasculogenesis and angiogenesis (Dorfman *et al.*, 1992; Drake *et al.*, 1997; Gering *et al.*, 1998; Liao *et al.*, 1998). The mutation of a single GATA site or a single EBS results in the complete loss of endothelial specificity of the minimal enhancer *in vivo*, whereas the mutation of either one of the two Scl/Tal-1 binding motives reduces reporter gene expression (Kappel *et al.*, 2000). Analysis of protein-DNA interactions on the *Vegfr2* enhancer suggests that all of these potential transcription factor binding sites are functional, as specific complexes are formed (Kappel *et al.*, 2000). DNase footprinting experiments demonstrate an EC-specific interaction of nuclear proteins with the EBS. However, transactivation studies indicate that Ets1 and Ets2 can not activate the *Vegfr2* enhancer *in vitro*.

Taken together, the DNA sequences of the 5′ flanking region of *VEGFR1* and *VEGFR2* genes have only a few common features, indicating that these genes are likely to be differentially regulated. Also the hypoxic induction of these two genes is mediated via different transcription factors. *VEGFR1* transcription is upregulated via the HIF-1α binding site present within the promoter region, while *Vegfr2* promoter driven transcription is stimulated by HIF-2α in hypoxic conditions (Gerber *et al.*, 1997; Kappel *et al.*, 1999). HIF-2α and Vegfr2 are co-expressed in postnatal mouse brain capillaries (Elvert *et al.*, 2002). Recently, HIF-2α has been shown to bind to two HRE-related sequences or HIF-2 binding sites (HBSs) located in close proximity to EBSs in the *Vegfr2* promoter (Fig. 6). HIF-2α and Ets1 interact physically with each other and stimulate the *Vegfr2* promoter activity in a synergistic manner *in vitro* (Elvert *et al.*, 2002).

2.1.3 The *VE-cadherin*, *PECAM-1* and *VWF* genes

VE-cadherin

Endothelial cells use cell adhesion molecules such as cadherins, selectins and integrins to attach themselves to one another and to the parenchyma. Cadherins are localized to structures known as adherens junctions. Although at least 3 cadherins have been found to be expressed in ECs, vascular endothelial (VE)-cadherin is the only one expressed specifically in ECs in various types of endothelia. VE-cadherin is expressed already at the very earliest stages of vascular development (E7.5 in mouse) in the blood islands and allantois of mouse embryos and it continues to be expressed in the vascular endothelium of most of the organs during development and in adulthood (Breier *et al.*, 1996). In contrast to most endothelial markers, it is not expressed in blood cells or in hematopoietic precursors. In embryonic stem cell-derived embryoid bodies, *VE-cadherin* null mutation inhibits the assembly of vascular-like structures, suggesting that VE-cadherin has an important role in vascular morphogenesis (Vittet *et al.*, 1997). Furthermore, gene targeting studies have revealed the vital role of VE-cadherin in vascular development, as inactivation of *VE-cadherin* gene results in early lethality of embryos with absence of differentiation of blood islands of the yolk sac into a primary vascular plexus (Carmeliet *et al.*, 1999; Gory-Faure *et al.*, 1999).

Approximately 2.5 kbp of the mouse *VE-cadherin* promoter region is sufficient to drive the expression of a reporter gene specifically in the ECs of transgenic mice (Gory *et al.*, 1999). Analysis of the proximal region of the promoter revealed the presence of three regulatory regions; one acts as a ubiquitous promoter and two regions silence the transcriptional activity in non-endothelial cells (Gory *et al.*, 1999) (Fig. 7). *VE-cadherin* promoter lacks TATA and CAAT boxes but has a GT box in proximity of a unique transcriptional start site (Gory *et al.*, 1998; Huber *et al.*, 1996). GT box is a consensus binding site for the Sp1 family of transcription factors. Sp1 is able to recruit TATA-binding protein and associated factors to the initiation complex. DNA-protein interaction studies indicated that Sp1 and Sp3 formed complexes with the GT box of *VE-cadherin* promoter. GT motif is a major determinant of *VE-cadherin* promoter activity as mutation of it caused 80% reduction in transcriptional activity. In addition, five EBSs were identified in the *VE-cadherin* promoter by sequence analysis. Two of these were determined to be essential for *VE-cadherin* promoter activity, as site-directed mutagenesis of either one of the EBSs reduced *VE-cadherin* promoter driven reporter gene expression dramatically in cultured cells (Gory *et al.*, 1998). Based on EMSA experiments, Erg forms a complex with one of the essential EBSs while Ets1 binds to both (Gory *et al.*, 1998; Lelièvre *et al.*, 2000). Overexpression of Ets1 resulted in the upregulation of VE-cadherin expression, thereby reducing EC density at confluence via its association with beta-catenin (Lelièvre *et al.*, 2000).

Platelet endothelial cell adhesion molecule-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) is a transmembrane protein belonging to the Ig gene superfamily. PECAM-1 has been widely used as a ubiquitous endothelial marker as it is highly expressed in ECs. However, also platelets, a subgroup of leukocytes, and hematopoietic precursors are PECAM-1 positive. PECAM-1 has important functions in angiogenesis, vascular injury repair, and control of the leukocyte extravasation.

The 5' flanking region of the human *PECAM-1* gene has been isolated and studied in cultured cells (Almendro *et al.*, 1996; Gumina *et al.*, 1997) (Fig. 7). An Alu sequence is located at the 5′-end of the promoter which lacks a consensus TATA box and CAAT element. Several transcription initiation sites were identified up to 601 bp upstream from the translation start site (Almendro *et al.*, 1996; Gumina *et al.*, 1997; Kirschbaum *et al.*, 1994). Many putative cis-acting control elements are present within the 5′ flanking region of *PECAM-1* gene. These include several EBSs, GC-rich sequences, GATA sites, an Sp1 site, and an AP-2 binding site (Fig. 7). Two continuous fragments driving gene expression either to the myeloid lineage (0.22 kbp *Nhe*I-*Bgl*II) or to the ECs (0.44 kbp *Bgl*II-*Pst*I) were identified from the promoter (Almendro *et al.*, 1996). The 0.22 kbp fragment exhibited PMA inducibility in myeloid cells and contained a PMA-responsive element recognized by Sp1 and EGR-1 transcription factors. Mutational analysis showed that a certain NF-κB site and one of the GATA sites are important for *PECAM-1* promoter activity in cultured cells (Botella

et al., 2000; Gumina *et al.*, 1997). Results from EMSA experiments have given further support for the functionality of these sites, as NF-κB and GATA2 were able to bind to their respective recognition sequences of the *PECAM-1* promoter (Botella *et al.*, 2000; Gumina *et al.*, 1997).

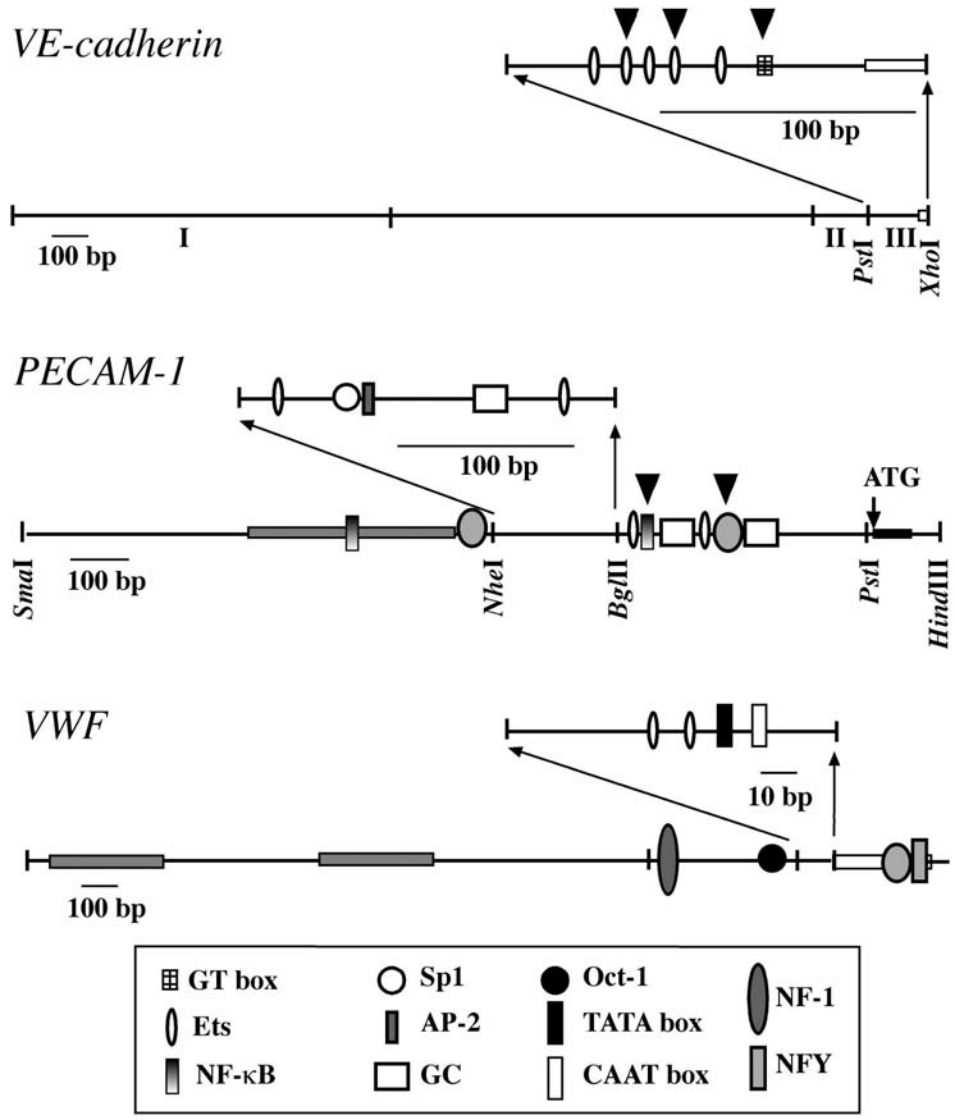

Figure 7. Schematic structure of *VE-cadherin*, *PECAM-1* and *VWF* regulatory regions. The three functional domains of *VE-cadherin* promoter sufficient to drive EC-specific expression *in vivo* are indicated as roman numbers (I, minor inhibitory region; II, major inhibitory region; III, basal promoter region). The basal promoter has one binding motif for Sp1/Sp3 factors, indicated as a GT box and several EBSs. Regulatory elements of *PECAM-1* are located in the 5′ flanking region, exon 1 and in the beginning of the first intron. The ATG translation start codon followed by the first exon (black box) is indicated. The human *VWF* promoter transcriptional activators located within the 90 bp core promoter are indicated in the figure. The critical transcription factor binding sites of *VE-cadherin* and *PECAM-1* promoters are pointed out by arrowheads while all the DNA elements of *VWF* promoter marked to the figure are important for its function. The Alu sequences present in *PECAM-1* and *VWF* promoters are represented as grey boxes**.**

von Willebrand factor

von Willebrand factor (vWF) is a multimeric glycoprotein that mediates adhesion of platelets to the underlying endothelium and serves as a carrier for the coagulation factor VIII. Expression of the *vWF* gene is restricted to the endothelium, megakaryocytes and platelets. vWF expression has been commonly taken as a proof for endothelial nature of a cell. However, vWF is not uniformly expressed in the endothelium and is therefore a marker of endothelial cell heterogeneity (Yamamoto *et al.*, 1998).

Both mouse and human *VWF* promoter regions have been isolated and they are highly homologous in both structure and function. Sequence analysis revealed the presence of TATA and CAAT boxes and the human *VWF* promoter has Alu sequences 5′ to the transcription initiation site (Assouline *et al.*, 1988). The results from transgenic mouse studies indicate that *VWF* gene is regulated in a modular fashion from one vascular bed to another. The 0.7 kbp fragment of human *VWF* promoter, including 0.5 kbp of the 5′ flanking sequence and the first exon, targeted reporter gene expression to a subpopulation of ECs in the yolk sac and to blood vessels of the brain (Aird *et al.*, 1995). A larger promoter fragment containing the 2.2 kbp 5' flanking sequence, the first exon, and the first intron directed the reporter gene expression also to the microvasculature of the heart and skeletal muscle (Aird *et al.*, 1997) (Fig. 7). Similar reporter gene expression pattern was detected with the regulatory regions of the mouse *vWf* gene located between the 2.6 kbp 5′ flanking region and the 3′-end of the first intron of the *vWf* (Guan *et al.*, 1999). The 5′ flanking regions and the first exons of the *VWF* genes have several conserved putative transcription factor binding sites including one GATA motif and two EBSs (Guan *et al.*, 1999; Jahroudi and Lynch, 1994; Schwachtgen *et al.*, 1997). Mutational analysis indicated that these DNA elements regulate *VWF* gene expression (Jahroudi and Lynch, 1994; Schwachtgen *et al.*, 1997). Of the Ets factors, Ets1 and Erg were able to transactivate a *VWF* core promoter construct, and EMSA studies further confirmed the interaction of these Ets factors with EBSs of *VWF* (Schwachtgen *et al.*, 1997). However, the core promoter is not sufficient to restrict *VWF* expression to ECs. Additional inhibitory regions containing NF-1, Oct-1 and NF-Y motifs are needed to repress *VWF* transcription in non-endothelial cells (Jahroudi *et al.*, 1996; Peng and Jahroudi, 2002; Schwachtgen *et al.*, 1998). DNA-protein interaction studies suggest that these repressors have inhibitory functions on the *VWF* promoter in all cell types, but ECs have a mechanism to overcome the inhibition (Peng and Jahroudi, 2002).

2.1.4 Other genes predominantly expressed in endothelial cells

The 5′ flanking regions of many other genes predominantly expressed in ECs have been partially characterized. In most cases, the regulatory elements have been studied only in cultured cells and are not discussed here. However, the results from studies on EC-specific regulatory elements targeting vascular endothelium also *in vivo* are briefly summarized here.

Endoglin

Endoglin (ENG) is a cell surface component of TGF-ß receptor complex highly expressed by ECs and in lesser amount on some hematopoietic cells, syncytiotrophoblasts of placenta and stromal cells (Ríus *et al.*, 1998). The 3.3 kbp *ENG* promoter lacks consensus TATA and CAAT boxes, but contains two GC-rich regions and a consensus Sp1 site near the transcriptional start sites (Ríus *et al.*, 1998). An Alu sequence was found along the 5′ region of the *ENG* promoter. 741 bp fragment of *ENG* promoter mediates EC-specific transcription in cultured cells and also drives expression in murine vascular endothelium (Ríus *et al.*, 1998). Several potential binding sites for Ets, GATA, AP-2, NF-κB and Sp1 transcription factors are present within this promoter region. Studies on cultured cells indicate that Ets proteins regulate *ENG* promoter activity. Mutation in one of the EBSs dramatically reduced the promoter activity compared with that of the WT promoter. Also a specific interaction of a member of the Ets family with this particular EBS was demonstrated by using EMSA (Ríus *et al.*, 1998).

Endothelin-1

Endothelin (ET-1) is critical for cardiovascular development in the embryo and for vascular homeostasis in the adult. It is expressed in ECs and in epithelial cells of the branchial arches during development. Both mouse and human *ET-1* promoters have been isolated. The 5.9 kbp 5′ flanking fragment of mouse *ET-1* directs gene expression to the endothelial, epithelial and mesangial cells (Harats *et al.*, 1995). Human *ET-1* promoter has a TATA box, and binding sites for AP-1 and GATA transcription factors are required for the expression of the *ET-1* gene (Lee *et al.*, 1999). Several members of the GATA family (GATA1, GATA2, and GATA3) and HIF-1α can transactivate the *ET-1* gene (Kawana *et al.*, 1995; Lee *et al.*, 1991; Yamashita *et al.*, 2001). Recently, a vascular endothelial zinc finger 1 (Vezf1) transcription factor has been shown to transactivate the *ET-1* promoter (Aitsebaomo *et al.*, 2001). Vezf1 expression is restricted to vascular endothelium and it is therefore an attractive candidate for a potential regulator of *ET-1* gene expression *in vivo* (Aitsebaomo *et al.*, 2001; Xiong *et al.*, 1999).

Intercellular adhesion molecule-2

Intercellular adhesion molecule-2 (ICAM2) is constitutively expressed on all vascular ECs and megakaryocytes as well as in some blood cells (de Fougerolles *et al.*, 1991). A 0.33 kbp 5′ flanking region of the *ICAM2* promoter confers EC-specific activity *in vitro* and *in vivo* (Cowan *et al.*, 1996; Cowan *et al.*, 1998). Sequence analysis revealed the presence of several DNA elements within this region; including an NF-κB and an Sp1 site, two GATA sites, four EBSs and an octamer, also present in the *Tie1* promoter and *Tie2* enhancer (Cowan *et al.*, 1998). Results from mutational and gel shift analyses demonstrate that the Sp1 site and the GATA sites function as positive regulatory elements in cultured ECs (Cowan *et al.*, 1998). Transactivation studies indicate that GATA1 and GATA2 are able to activate the *ICAM2* promoter (Cowan *et al.*, 1998). Furthermore, site-directed mutagenesis of the *ICAM2* promoter implicated that the octameric sequence, the NF-κB site and three consensus EBSs

are needed for basal activity (Cowan *et al.*, 1998; McLaughlin *et al.*, 1999). Two of these EBSs are involved in TNFα-induced down-regulation of ICAM2 expression. Interestingly, TNFα stimulation of cultured ECs reduces also Erg trancription factor expression, which is able to transactivate *ICAM2* promoter (McLaughlin *et al.*, 2001; McLaughlin *et al.*, 1999).

Endothelial nitric oxide synthase

Endothelial nitric oxide synthase (eNos) is an enzyme responsible for generating nitric oxide and it is expressed predominantly in the endothelium. eNos has an important function in regulating vascular tone and remodeling (Huang *et al.*, 1995; Rudic *et al.*, 1998). The 1.6 kbp region of the human *eNOS* promoter targets reporter gene expression mainly to ECs both *in vitro* and *in vivo* (Guillot *et al.*, 1999; Guillot *et al.*, 2000; Zhang *et al.*, 1995). However, this promoter drives reporter gene expression only to a subpopulation of ECs within the heart, skeletal muscle, brain and aorta, although the endogenous gene is more broadly expressed, indicating that all the DNA elements needed to recapitulate *eNOS* gene expression are not present within the 1.6 kbp fragment (Guillot *et al.*, 1999; Guillot *et al.*, 2000). The *eNOS* promoter is TATA-less and transfection studies in cultured cells by using various *eNOS* promoter constructs indicate that Sp1 and GATA sites are important for promoter activity (Zhang *et al.*, 1995). EMSA experiments indicate that Sp1 and GATA2 transcription factors are able to bind to these target sites *in vitro* (Zhang *et al.*, 1995).

2.2 Transcription factors regulating endothelial gene expression

Characterization of the regulatory regions of genes that are specifically expressed in ECs is a useful starting point for gaining information about the mechanisms that underlie endothelial differentiation and maturation. However, it is clear that the expression of even a single gene is regulated by several transcription factors belonging to different families. Gene expression is regulated also temporally and spatially, depending on which transcription factors are present at certain time and tissue. In addition to synthesis, the activity of transcription factors may be controlled by modification, presence of the ligand or availability in the nucleus. As all of these regulatory events may participate in regulating the transcription of a single gene, it is evident that our knowledge about the expression of endothelial genes is still rather limited. However, comparison of transcription factor binding sites between different genes predominantly expressed in ECs indicate that EBSs and GATA sites are major regulators of promoter/enhancer activity in most of the cases. Therefore, the transcription factors binding to these DNA elements are discussed below.

2.2.1 Ets factors

Ets transcription factor family includes over 50 members characterized by a conserved sequence of approximately 85 aa named the Ets domain, which is responsible for DNA-binding (reviewed in Lelièvre *et al.*, 2001). The Ets domain folds into a winged helix-turn-helix motif and binds to a consensus DNA sequence GGAA/T, named Ets binding site or EBS (Donaldson *et al.*, 1994; Karim *et al.*, 1990). The sequences flanking the EBS are variable and may affect the

binding specificity of Ets factors. Ets factors bind DNA generally as monomers but they can also associate with other transcription factors bound to their cognate motifs in the vicinity of the EBS (Wasylyk *et al.*, 1993). Ets proteins are implicated in the regulation of gene expression during many important biological processes, such as cell growth, differentiation and transformation. Most Ets proteins activate transcription except for Erf (Ets2 repressor protein), Net and Tel, which function as inhibitors (Lopez *et al.*, 1999; Maira *et al.*, 1996; Sgouras *et al.*, 1995). The function of some Ets factors is regulated by phosphorylation of certain Thr or Ser residues as the result of MAPK or PI3K pathway stimulation by activated oncogenes or receptor-mediated tyrosine kinases (Yordy and Muise-Helmericks, 2000).

EBSs are found in the regulatory regions of genes that encode growth factors, oncoproteins, transcription factors and MMPs (reviewed in Lelièvre *et al.*, 2001). Ets1 is the founding member of the ETS family. Studies on cultured cells suggest that *VEGFR1*, *Vegfr2*, *VE-cadherin* and *VWF* are directly regulated by Ets1 transcription factor (Dube *et al.*, 1999; Gory *et al.*, 1998; Kappel *et al.*, 2000; Lelièvre *et al.*, 2000; Schwachtgen *et al.*, 1997; Wakiya *et al.*, 1996). Ets1 is highly expressed by endothelial precursors during vasculogenesis and in ECs during angiogenesis (reviewed in Lelièvre *et al.*, 2001). However, Ets1 expression is not restricted to the endothelium since its mRNA is also found e.g. in the trophoblasts, somites and migrating neural crest cells during embryogenesis and in stromal fibroblasts of invasive tumors (Bolon *et al.*, 1996; Fafeur *et al.*, 1997; Luton *et al.*, 1997; Wernert *et al.*, 1994). Ets1 increases the mRNA levels of angiogenesis-related molecules such as MMPs, converting ECs to an active state (Oda *et al.*, 1999). Overexpression of Ets1 in the ECs, but not in fibroblasts, decreased cell density at confluence (Lelièvre *et al.*, 2000). Loss of function experiments failed to address Ets1 role during vascular development, probably due to the redundant function of other members of this transcription factor family expressed in ECs (Barton *et al.*, 1998).

Ets2 has been shown to activate *VEGFR1* and *Vegfr2* promoters *in vitro* (Dube *et al.*, 1999; Kappel *et al.*, 2000; Wakiya *et al.*, 1996). However, Ets2 is not expressed in ECs *in vivo,* indicating that it does not at least directly regulate EC-specific gene expression (Yamamoto *et al.*, 1998). Ets2 expression is detected in many other cell types, in the developing limb buds and tail region during the early embryonic development (Maroulakou *et al.*, 1994). Later, Ets2 is expressed in most organs including lung, skin, gut and brain (Kola *et al.*, 1993; Maroulakou *et al.*, 1994). Ets2 deficient mice die at E8.5 due to severe defects in extraembyonic tissues (Yamamoto *et al.*, 1998). If the extraembryonic defects are rescued by aggregation with tetraploid mouse embryos, Ets2 deficient mice are viable and show defects only in hair development (Yamamoto *et al.*, 1998). Interestingly, tumors grown in heterozygous female mice carrying one defective *Ets2* allele were one-half the size of tumors grown in WT mice. These results suggest that Ets2 regulates tumor progression possibly via MMPs, and may thus regulate the expression of endothelial genes indirectly (Neznanov *et al.*, 1999).

Studies on cultured cells indicate that Erg regulates *VEGFR1, VE-cadherin*, *VWF* and *ICAM-2* expression (Gory *et al.*, 1998; McLaughlin *et al.*, 1999; Schwachtgen *et al.*, 1997; Wakiya *et al.*, 1996). During mouse embryogenesis, Erg is expressed in mesodermal tissues, including the endothelial, precartilaginous and urogenital areas as well as in migrating neural crest cells (Vlaeminck-Guillem *et al.*, 2000). Decrease of Erg expression by antisense treatment results in decreased EC proliferation and tube formation, whereas injection of Xenopus Erg into Xenopus embryos was shown to lead to eye malformations and ectopic EC differentiation (Baltzinger *et al.*, 1999; McLaughlin *et al.*, 2001). These results indicate that Erg is involved in blood vessel formation and it is therefore an attractive candidate for a direct transcriptional regulator of endothelial genes *in vivo*. In addition, Erg regulates the transcription of genes encoding for extracellular proteolytic enzymes, and it inhibits the Ets2-mediated activation of the *MMP3* promoter, indicating that it may modulate angiogenesis by several ways (Buttice *et al.*, 1996).

The other Ets factors that have been detected so far in cultured ECs are Fli1, Nerf, ERM, ER81, and Tel. ERM and ER81 mRNAs have not been detected in ECs during mouse embryogenesis, indicating that these transcription factors are not direct regulators of EC-specific transcription of genes (Chotteau-Lelièvre *et al.*, 1997). On the other hand, Fli1, Nerf (discussed in Results and Discussion section, pages 46-47), and Tel appear to have important functions during angiogenesis. Fli1 is closely related to Erg in structure and it is expressed mainly in hematopoietic cells and ECs. Fli1 has an almost overlapping expression pattern with Erg during embryonic development (Mélet *et al.*, 1996; Vlaeminck-Guillem *et al.*, 2000). *Fli1* gene disruption in mice leads to embryonic death at E12.5 due to hemorrhages and impaired hematopoiesis (Hart *et al*., 2000; Spyropoulos *et al.*, 2000). Fli1 is a target gene for Ets1 and overexpression of Fli1 activates the *VE-cadherin* promoter (Lelièvre *et al.*, 2002). Interestingly, Tie2 levels in Fli1-deficient embryos were downregulated in comparison to their heterozygous littermates, suggesting that Fli1 regulates Tie2 expression *in vivo* (Hart *et al.*, 2000). Tel is able to form homodimers, which is necessary for its activity (Lopez *et al.*, 1999). In addition, Tel is able to bind Fli1 and to inhibit Fli1-mediated transactivation (Kwiatkowski *et al.*, 1998). Targeted disruption of Tel results in defective angiogenesis in the embryonic yolk sac, in intra-embryonic apoptosis of mesenchymal and neural crest cells and in embryonic death at E10.5-11.5 (Wang *et al.*, 1997).

2.2.2 GATA factors

The GATA transcription factors play an important role in the regulation of a number of hematopoietic and endothelial genes. So far, six GATA transcription factors have been identified in vertebrates. They recognize a consensus sequence motif, (G/A)GATA(A/T), through a conserved zinc finger DNA binding domain (Orkin, 1992). GATA1, GATA2 and GATA3 are prominently expressed in hematopoietic stem cells, while GATA4, GATA5, and GATA6 are expressed in mesodermally and endodermally derived tissues. Gene inactivation studies of GATA factors failed to show a vascular phenotype in mice. Results indicated that GATA1, GATA2 and GATA3 are master regulators of hema-

topoietic and lymphoid cell development (Ho *et al.*, 1991; Joulin *et al.*, 1991; Ko *et al.*, 1991; Pevny *et al.*, 1991; Tsai *et al.*, 1994; Tsai *et al.*, 1989). GATA4 null mice die around E8-E9 due to defects in heart development and ventral closure of the foregut (Kuo *et al.*, 1997a; Molkentin *et al.*, 1997). This phenotype results from defects in the folding of the embryo needed for normal cardiac development. GATA5 is largely dispensable for embryonic development, as the only phenotype resulting from targeted disruption of the GATA5 gene in mice is defective genitourinary tract development in females (Molkentin *et al.*, 2000b). GATA6 deficient mice die early during development, at E6.5-E7.5 due to defects in visceral endoderm function (Koutsourakis *et al.*, 1999; Morrisey *et al.*, 1998). Though GATA factors are broadly expressed, it has been speculated that they regulate tissue-specific gene expression in multiple cell types through unique interactions with other transcription factors (Molkentin *et al.*, 2000a).

GATA factors participate in the regulation of *PECAM-1*, *VWF, ICAM2, endothelin-1*, *eNOS*, and *P-selectin* promoter and *Vegfr2* enhancer activities (Botella *et al.*, 2000; Cowan *et al.*, 1998; Gumina *et al.*, 1997; Jahroudi and Lynch, 1994; Kappel *et al.,* 2000; Kawana *et al.*, 1995; Pan and McEver, 1993; Zhang *et al.*, 1995). In addition to hematopoietic cells, GATA2 is abundantly expressed in ECs, and it is therefore the best candidate for a GATA factor to regulate EC-specific gene expression (Dorfman *et al.*, 1992; Orkin, 1992). Furthermore, recent studies indicate that GATA2 motif, together with EBSs located within a 3′ enhancer element of the *Scl/Tal1* gene, target *Scl/Tal1* transcription to hemangioblasts (Göttgens *et al.*, 2002). Scl/Tal1 is a transcription factor that regulates VEGFR-2 expression (Kappel *et al.*, 2000). VEGFR-2 is the first endothelial marker to be expressed in mesodermal progenitor cells. Therefore, the characterization of the upstream pathways regulating VEGFR-2 expression may provide information about hemangioblast commitment (Choi *et al.*, 1998). Scl/Tal1 specifies vascular progenitors in zebrafish and its overexpression increases the number of VEGFR-2 expressing endothelial precursor cells (Gering *et al.*, 1998; Liao *et al.*, 1998). Although yolk sac vascularization is defective in Scl/Tal1 null mice, endothelial cell differentiation proceeds normally. Rescue of the hematopoietic defects by using the regulatory elements of GATA1 transcription factor indicated that Scl/Tal-1 is dispensable for vascular cell specification but essential for angiogenic remodeling of the yolk sac capillary network into complex vitelline veins (Visvader *et al.*, 1998).

Taken together, it appears that none of the Ets or GATA factors characterized so far is the major common regulator of endothelial gene expression. Whether such general regulator for ECs exists, is not known yet. So far, only one transcription factor, Vezf1, is known to be expressed in an EC-restricted manner (Xiong *et al.*, 1999). However, the only downstream target identified for Vezf1 is endothelin-1 (Aitsebaomo *et al.*, 2001). Another potential transcription factor regulating EC-specific gene expression is lung Kruppel-like factor (LKLF). It is predominantly expressed in lung ECs, but no changes in *Tie1*, *Tie2* or *PDGF-B* expression levels were seen in LKLF-deficient embryos, indicating that it does not regulate the expression of these genes (Kuo *et al.*, 1997b). Also, it

is possible that ECs have specific co-factors interacting with ubiquitous/semi-restricted transcription factors, resulting in tissue-specific transcription of genes. However, comparison of the regulatory regions of EC-specific genes suggests that there are several different mechanisms to target gene expression specifically to endothelial cells. Further studies are needed to reveal these complicated but fundamental issues.

3 USE OF ENDOTHELIAL TARGETING ELEMENTS IN FUTURE GENE THERAPY

The regulatory regions driving gene expression specifically to the lymph/angiogenic ECs may have potential in the generation of targeting vectors for gene therapy of vascular diseases. As ECs line the lumen of blood vessels, they form a potentially useful target for gene therapy for several reasons. First, vascular endothelium has a major role in regulating important biological processes such as tumor angiogenesis. Second, endothelium covers a large surface area in proximity to the circulation. Third, in tumors, ECs are less prone to developing genetic variants resistant to anti-angiogenic tumor therapy than tumor cells (Kerbel, 1997). Furthermore, because angiogenesis does not occur in the adult except during times of wound healing, inflammation, ovulation and pregnancy, it is possible to develop therapies with minimal toxicities.

The expression of at least most of the EC-specific genes is not ubiquitous in all types of endothelial cells, indicating that transcriptional control mechanisms differ between subpopulations of endothelial cell lineage. Therefore, choice of the best promoter to be used in therapeutic stimulation or inhibition of angiogenesis depends on the target tissue. In fact, one of the bottlenecks in the identification of regulatory elements needed for targeting gene expression to ECs has been the lack of systematic dissection of the expression patterns driven by a certain regulatory region. Also, as most of the studies on EC-specific promoters have focused on a single promoter, comparison of the activities of different EC-specific promoters for a certain application is difficult. However, recently studies comparing the reporter gene activities driven by different endothelial regulatory regions have been published (He *et al.*, 2001; Marchetti *et al.*, 2002; Nicklin *et al.*, 2001). Furthermore, tumor vessels are heterogeneous and tumor angiogenesis results in differential activation of EC-specific promoters (Minami *et al.*, 2002). In some cases, the basic mechanism involved in the interaction of tumor cells and endothelium may provide clues for choosing the best promoter. EC surface molecules may be involved in metastasis of different types of tumor cells. Unfortunately, in many tumors the mechanism that facilitates the migration of circulating tumor cells into tissues is unresolved. Recently, use of the *in vivo* phage display method has demonstrated that tumor vascular ECs express a specific range of molecules (Pasqualini *et al.*, 2000). This technology may provide tools for specific targeting of blood vessels in primary tumors or metastases, as well as other disease-related endothelia. Several reports indicate that in some cancers, blood channels may be lined by cancer cells rather than by ECs, although these findings still need to be confirmed

(Pasqualini *et al.*, 2002). If this kind of vascular mimicry exists in tumors, it may affect the efficacy of EC-targeted gene therapy.

In addition to generating ways to target specific tissues and organs, there are a number of other open questions to be answered before successful gene therapy can be performed. These problems are related to the control of the efficiency and duration of gene expression, induction of immune reactions and possible cytotoxicity of vectors. Also, it has to be kept in mind that the promoter activity may change when the promoter is ligated into viral vectors often used to target ECs (Nicklin *et al.*, 2001). Therefore, several *ex vivo* and *in vivo* experiments are needed to confirm the expression pattern driven by a certain regulatory region after ligation to a viral vector. Despite these concerns, studies on tumor cell lines and on mice indicate that adenovirus-based vectors containing either *VEGFR1* or *preproendothelin* promoters may provide useful tools to target tumor endothelium (Bauerschmitz *et al.*, 2002; Nicklin *et al.*, 2001; Varda-Bloom *et al.*, 2001). Therefore, regulatory regions which target specifically ECs *in vivo* are not anymore the major limiting factor for the generation of therapeutic strategies to vascular diseases. However, generation of both efficient and safe endothelial gene targeting vectors for future gene therapies still remains both a challenge and an opportunity.

AIMS OF THE PRESENT STUDY

This study was undertaken to obtain information about the transcriptional regulation of *Tie1* and *Vegfr3* genes. *Tie1* promoter region targeting expression of the *LacZ* gene specifically to ECs *in vivo* had been previously identified, while nothing was known about the *Vegfr3* promoter. The specific aims of the study were:

1. Characterization of DNA elements needed for *Tie1* promoter activity and cell type specificity. Our goal was to identify transcription factors that may regulate *Tie1*. To study the use of *Tie1* promoter in future gene therapy to target expression of soluble proteins into the blood circulation, we generated transgenic mice expressing human growth hormone cDNA under the control of *Tie1* promoter.

2. Generation of fluorescent *Tie1* reporter mice to allow monitoring of vascular development *in vivo* and isolation of primary mouse endothelial cells**.** Additionally, as the regulatory elements of EC-specific genes may have therapeutic applications, we also analyzed *Tie1* transcription in tumor endothelium.

3. Identification and characterization of the *Vegfr3* promoter region. In particular, we were interested in finding out which region is required to drive gene expression specifically in lymphatic endothelium. As mutations in *VEGFR3* gene have been linked to hereditary lymphedema, we wanted to characterize the genomic organization of the *VEGFR3*.

MATERIALS AND METHODS

1. Characterization of DNA elements needed for *Tie1* promoter activity and cell type specificity (I)

To identify *Tie1* RNA transcription initiation sites, RNase protection and primer extension assays were performed by analyzing RNA isolated from mouse embryos (E12) and adult kidneys. It was known from a previous study that 0.8 kbp *Tie1* promoter confers EC-specific reporter gene expression *in vivo* (Korhonen, 1995). In order to study which of the conserved putative transcription factor binding sites present in the *Tie1* promoter are needed for its activity and cell type specificity, the promoter was cloned into a luciferase reporter vector. Progressive deletions and site-directed mutations were made to the promoter and the effect of these was studied in transfected cells by using a luciferase reporter assay. Both endothelial and non-endothelial cells were used. Because a number of EBSs are conserved between the mouse and human *Tie1* promoters, the transactivation capacity of seven transcription factors of the Ets family was analyzed. To determine DNA sequences critical for EC-specific expression of *Tie1 in vivo*, transgenic mice having a *LacZ* reporter driven by mutated *Tie1* promoter fragments were produced and analyzed for ß-galactosidase activity at E11.5. To study *Tie1* promoter driven secretion of protein into the circulation, transgenic mice expressing the human growth hormone cDNA under the control of *Tie1* promoter were generated. The level of hGH in the serum was determined by using a hGH immunoradiometric assay.

2. Generation of fluorescent *Tie1* reporter mice to allow monitoring of vascular development and endothelial cell isolation (II)

To produce transgenic reporter mice expressing green fluorescent protein in the vascular endothelium, EGFP or a fusion protein consisting of a Zeosin resistance marker and EGFP (ZEGFP) was expressed under the control of the *Tie1* promoter. The reporter gene expression in *Tie1*-Z/EGFP mouse lines was studied at E10.5 by fluorescence microscopy. Mouse lines expressing high levels of the transgene were studied further by analyzing the transgene expression at several stages during embryogenesis and in adulthood. The Z/EGFP reporter expression pattern in *Tie1*-Z/EGFP mice was compared with the LacZ reporter expression pattern in *Tie1*-LacZ and *Tie1+/LacZ* mice, having the same promoter fragment or the endogenous *Tie1* locus driving the LacZ expression, respectively (Korhonen *et al.*, 1995; Puri *et al.*, 1995). In addition to fluorescence microscopy (Z/EGFP) and ß-galactosidase staining (LacZ), reporter gene expression in adult organs of *Tie1*-Z/EGFP, *Tie1*-LacZ and *Tie1+/LacZ* mice was studied by Northern blot analysis and RT-PCR. To analyze *Tie1* expression in different types of vessels in adults, several organs from *Tie1*-LacZ and *Tie1+/LacZ* mice were studied further after vascular perfusion with biotin-labeled lectin and ß-galactosidase staining. As the *Lycopersicon esculentum* lectin binds to the surface of the ECs after intravenous injection, all blood ves-

sels can be visualized. To study whether *Tie1* is expressed in the lymphatic vessels, the *Tie1+/LacZ* and *Tie1*-LacZ mice were mated with the K14-VEGFR-3-Ig and K14-VEGF-C156S mice having lymphatic phenotypes (Mäkinen *et al.*, 2001a; Veikkola *et al.*, 2001).

To confirm the endothelial cell type specific expression of EGFP in *Tie1*-Z/EGFP embryos, cells isolated at E9.5 were allowed to attach on glass coverslips and were stained with antibodies against CD31 (MEC13.3, PharMingen, San Diego, CA), VEGFR-2 (AVAS12α1, PharMingen) and VE-cadherin (11D4.1, PharMingen). EGFP and CD31 expression was analyzed by flow cytometry. EGFP-positive cells were isolated by FACS. Cells from the positive pool were resuspended in culture medium, plated on gelatin and expanded in culture. The expression of CD31, VEGFR-2 and VEGFR-3 (Kubo *et al.*, 2000) was studied by immunofluorescence stainings. EGFP, *Tie1* and *Tie2* expression was followed also by Northern blot analysis. To produce endothelial cell lines, cells prepared from *Tie1*-ZEGFP embryos at E12 were immortalized by retroviral infection transfecting polyoma middle T-antigen and neomycin resistance selection marker into the cells. Selection with neomycin (800 μg/ml) alone and with various concentrations of Zeosin ranging from 100 μg/ml to 1.5 mg/ml was started 72 h after the infection. Noninfected embryonic cells were used as controls.

To analyze *Tie1* promoter activation in pathological angiogenesis, Lewis lung carcinoma (LLC) cells were injected subcutaneously into the backs of *Tie1*-Z/EGFP and *Tie1*-LacZ transgenic mice. For comparison, tumors grown in the *Tie1+/LacZ* mice were analyzed. Tumors grown in *Tie1*-Z/EGFP mice were analyzed directly by fluorescence microscopy whereas tumors grown in *Tie1*-LacZ transgenic and *Tie1+/LacZ* heterozygous mice were stained for ß-galactosidase activity, followed by sectioning and counterstaining with nuclear fast red.

3. The genomic structure and regulatory region of *VEGFR3* (III)

To characterize the exon-intron boundaries of *VEGFR3*, a genomic cosmid clone containing the entire sequence was subcloned and partially sequenced. Polymorphic variation in the *VEGFR3* gene was identified by resequencing more than 50 chromosomes and allele frequencies were estimated from the sequence results. To identify the *VEGFR3* promoter region, the 5′ flanking regions of human and mouse *VEGFR3* genes were isolated, sequenced and compared to each other. To analyze whether the upstream sequences of *Vegfr3* confer promoter activity, a 3 kbp fragment 5′ to the first coding exon was cloned into a luciferase reporter vector. In addition, progressive 5′-deletions were made to the *Vegfr3* promoter driving luciferase gene expression to find out critical sequences for its activity. Constructs were transfected in cultured endothelial and nonendothelial cells to study if *Vegfr3* promoter confers cell type specific transcription. Cell extracts were assayed for luciferase activity. For *in vivo* analysis, transgenic mouse embryos having either the 3.6 kbp, 1.6 kbp or 0.8 kbp *Vegfr3* promoter fragment upstream of the *LacZ* reporter gene were generated. The em-

bryos were genotyped by PCR analysis of amnion DNA and stained for ß-galactosidase expression at E15.5. At that developmental stage, the lymphatic vessels are developing, while the skin is still permeable to the ß-galactosidase staining reagents.

RESULTS AND DISCUSSION

1. DNA elements needed for the *Tie1* promoter activity and cell type specificity (I)

RNase protection analysis from both embryonic and adult kidney RNAs using two riboprobes produced a doublet of bands having corresponding 5′ ends. The product of primer extension analysis had the corresponding 5′ end as the RNase protection product, indicating that *Tie1* promoter has a single major transcription start site. *Tie1* promoter activity was studied in endothelial and nonendothelial cell lines and the results indicated that the activity of the promoter is relatively specific for ECs. Deletion of the 5′ end including the GT-repeat area and single EBS did not markedly change *Tie1* promoter activity, suggesting that these sites are dispensable for promoter activity. Studies of *Tie1* promoter-*LacZ* transgenic embryos gave further support for this view, as some transgenic embryos showed reporter gene expression specifically in ECs when these sequences were deleted. In contrast, deletion of the 3′ end of the *Tie1* promoter, including the transcription initiation site as well as mutations made to the Ets doublet sites, to the AP-2 site or to the octamer sequence, reduced *Tie1* promoter activity dramatically in cultured cells. Interestingly, certain DNA elements present within the *Tie1* promoter are found in regulatory regions of other mainly endothelial genes. Similar octameric sequences are found in the *Tie2* enhancer and in the *ICAM2* promoter (Cowan *et al.*, 1998; Schlaeger *et al.*, 1997). Whereas a mutation destroying the *Tie2* octamer mainly increased integration site dependency of the reporter construct, mutation of the octamer sequence present in the *ICAM2* promoter reduced reporter activity by 70% in cultured ECs. Furthermore, transgenic embryos with mutated 3′ doublet EBS of the *Tie1* promoter had strongly reduced reporter gene expression, confirming the critical role of these EBSs for *Tie1* promoter activity. Ets factors are important regulators of *Tie2* gene as well. Deletion of the *Tie2* enhancer fragment containing solo and doublet EBSs completely abolished the *in vivo* activity of the enhancer (Schlaeger *et al.*, 1997). Also several other regulatory regions of genes predominantly expressed in ECs have EBSs, suggesting that Ets transcription factors are important for EC-specific transcription (Table 2).

Among the seven different members of Ets transcription factor family tested, NERF-2 showed the strongest (8.5 fold) transactivation of *Tie1* promoter *in vitro* in an EBS-dependent manner. NERF-2 is one of the three isoforms of NERF, which is a recently isolated member of the Ets factor family (Oettgen *et al.*, 1996). The other NERF isoform, NERF-1A, was not able to transactivate *Tie1* promoter. Among the Ets family, NERF has the highest amino acid sequence homology to ELF-1. Interestingly, ELF-1 is also able to transactivate *Tie1* promoter (Dube *et al.*, 2001). NERF and ELF-1 are involved in the regulation of several T and B cell specific genes (Oettgen *et al.*, 1996). Recent data indicates that NERF-2 and ELF-1 are also able to transactivate *Tie2* reporter constructs by 10 to 30 fold mainly via EBSs located in the proximal promoter (Dube *et al.*, 1999) (Fig. 5). Both NERF-2 and ELF-1 are expressed in at least a subset of ECs. Therefore they are good candidates for transcriptional regulators of *Tie*

genes *in vivo* (Dube *et al.*, 1999; Dube *et al.*, 2001). Recently, by using chicken (c) as a model, cNERF-2 expression has been reported to be high during the early development, particularly in the developing heart and limb (Gaspar *et al.*, 2002). *In situ* hybridization results indicate that cNERF-2 is expressed predominantly in the developing neural tube and in the endothelium of large vessels, while ELF-1 is also highly expressed in the capillaries (Dube *et al.*, 2001; Gaspar *et al.*, 2002). Surprisingly, in contrast to cELF-1 and human NERF-2, cNERF-2 does not transactivate *Tie* promoters, although it is able to bind to the *Tie1* and *Tie2* EBSs. Cotransfection experiments of cNERF2 and cELF-1 with *Tie1*- and *Tie2*-reporter constructs demonstrate that cNERF-2 functions as a competitive inhibitor of cELF (Gaspar *et al.*, 2002). These results suggest that a change in NERF-2 function from a negative to a positive regulator has occurred during evolution.

The other Ets factors tested for their ability to transactivate *Tie1* promoter were ETS-1, ETS-2, TEL, SAP-1 and ELK-1. Ets1, Ets2 and Tel have been shown to regulate angiogenesis (for discussion, see 2.2.1). SAP-1 and ELK1 interact with the serum response factor. Our results indicate that ETS-1 and ETS-2 are able to transactivate the *Tie1* promoter constructs maximally by three- to fourfold whereas the other Ets factors tested did not transactivate the *Tie1* promoter. None of these Ets factors could transactivate the *Tie2* promoter or enhancer (Dube *et al.*, 1999). Taken together, the results indicate that these transcription factors are not major regulators of Tie genes. Recently, a DNA microarray analysis has been performed from a mouse brain EC line either overexpressing Ets1 or not (Lelièvre *et al.*, 2002). *Tie1* and *Tie2*, as well as *Vegfr1* and *Vegfr2* gene expression profiles were similar in both cell lines, while *VE-cadherin* and *Ang2* expression levels were increased in Ets1 overexpressing ECs (Lelièvre *et al.*, 2002; Lelièvre *et al.*, 2000). In conclusion, although Ets1 is highly expressed in ECs during vasculogenesis and angiogenesis, it is probably not the major regulator of EC phenotype. A summary of Ets factors transactivating EC-specific genes is presented in Table 2.

Table 2. Endothelial Ets target genes.

Gene	Ets factor
Tie1	NERF-2 ELF-1 Ets1 Ets2
Tie2	NERF-2 ELF-1 Fli1
VEGFR1	Ets1 Ets2 Erg
Vegfr2	Ets1 Ets2
VE-cadherin	Ets1 Erg Fli1
ICAM2	Erg
VWF	Ets1 Erg

As angiogenesis is involved in many diseases, one goal in studying EC-specific promoters is to define elements that might be useful for pro- or antiangiogenic therapy. Transgenic mice expressing human growth hormone cDNA under the 0.8 kbp *Tie1* promoter were generated and analyzed as a model of

Tie1 promoter driven secretion of protein into the circulation. High amounts of hGH were detected in the circulation of transgenic mice and the hGH level correlated with the copy number of the transgene, indicating that the *Tie1* promoter may have potential for use in gene therapy.

2. Generation of fluorescent *Tie1* reporter mice to allow monitoring of vascular development and endothelial cell isolation (II)

Three founder lines showed fluorescence for EGFP and two for ZEGFP in the developing blood vessels of E10.5 embryos. Intravital monitoring demonstrated that the EGFP reporter recapitulates the *Tie1* expression pattern in the developing vasculature. The EGFP expression was not uniform throughout blood endothelia, as starting around midgestation, *Tie1* promoter activity was stronger in the arteries than in the veins. Surprisingly, despite bright fluorescence seen in the vessels of *Tie1*-Z/EGFP transgenic embryos, no EGFP signal could be detected in most of the tissues in adult mice. However, several tissues from adult *Tie1*-LacZ transgenic and *Tie1+/LacZ* heterozygotic mice showed ß-galactosidase staining. As each EGFP protein molecule represents one fluorophore, the fluorescence signal is not amplified as in the case of chromophore signal produced by the enzymatic ß-galactosidase reporter. In most tissues analyzed, staining was weaker for the *Tie1*-LacZ transgene than for the *Tie1+/lacZ* knock-in, indicating that some regulatory elements needed for endogenous *Tie1* expression in adult tissues are missing from the 0.8 kbp *Tie1* promoter. It is unknown whether additional *Tie1* enhancer elements are located in the first intron as in the case of *Tie2* and *Vegfr2* genes. The results of Northern blot analysis and RT-PCR confirmed the lack of EGFP and LacZ expression in most tissues of transgenic mice, although endogenous Tie1 mRNA was detected in all tissues analyzed. Altogether, our results indicate that the lack of fluorescence in adult *Tie1*-Z/EGFP mice is due to the lack of reporter gene expression.

Lectin perfused and ß-galactosidase stained skin from the ears of *Tie1+/LacZ* mice indicated *Tie1* transcription mainly in the arterioles and in the capillaries, although very faint and scattered expression could be detected in large arteries and veins. Similar blood vascular expression pattern was seen in the *Tie1*-LacZ transgenic mice, indicating that *Tie1* promoter targets mainly arterioles and capillaries in adult mice. Furthermore, analysis of adult skin indicated that *Tie1* transcription occurs also in the lymphatic endothelium. Similar reporter gene expression was seen in the lymphatic vessels of *Tie1*-LacZ and *Tie1+/LacZ* mice. The lymphatic nature of the stained vessels was confirmed by mating *Tie1*-LacZ transgenic and *Tie1+/LacZ* heterozygous mice with transgenic mice having lymphatic hypoplasia or hyperplasia. The K14-VEGFR-3-Ig adult mice lack lymphatic vessels of the skin, whereas the lymphatic vessels in the skin of the K14-VEGF-C156S transgenic mice are hyperplastic (Mäkinen *et al.*, 2001a; Veikkola *et al.*, 2001). No blue vessels without lectin staining were seen in the skin of *Tie1*-LacZ x K14-VEGFR-3-Ig mice, whereas in the *Tie1+/LacZ* x K14-VEGF-C156S mice, the lectin-negative

and ß-galactosidase-positive vessels were dilated, confirming that *Tie1* is transcribed in the lymphatic endothelium. Lymphatic *Tie1* transcription was not restricted to adult skin, as ß-galactosidase expression was seen also in the lymphatic endothelium of the diaphragm and esophagus, in the lymphatic vessels on surface of the heart and small intestine as well as in mesenteric lymphatic vessels. Interestingly, comparison of wholemount ß-galactosidase-stained *Tie1+/LacZ* and *VEGFR-3+/LacZ* embryos indicated that Tie1 is already expressed in the lymphatic endothelium during embryogenesis at E13.5. Further analyses are needed to characterize Tie1 expression and its function in lymphatic endothelium in detail. Our preliminary results from Tie1 deficient embryos at E12.5 indicate that lymphatic vessels do form, indicating that Tie1 is not needed for lymphatic differentiation or initial formation of the lymphatic vasculature (K. Iljin, unpublished results).

Flow cytometry using EGFP allowed the isolation of essentially pure *Tie1* expressing endothelial cells from transgenic mouse embryos. All EGFP-positive cells were also positive for CD31. CD31, VEGFR-2 and VEGFR-3 were expressed in such cultures at passage 2, although their expression decreased upon repeated subculturing. The results of Northern blotting confirmed that the cells at passages 4 and 8 express EGFP, *Tie1* and *Tie2*. The cellular morphology was endothelial-like with a typical cobblestone morphology at confluency. The cells retained EGFP positivity at least until passage 12. We were not able to produce stable endothelial cell cultures from the *Tie1*-ZEGFP embryos by retroviral infection of polyoma middle T-antigen. Whether this was due to (1) reduced *Tie1* promoter activity, (2) cytotoxicity of Z/EGFP, (3) environmental changes caused by the death of surrounding cells or (4) nonoptimal culture conditions was not determined. However, EC-cultures have been made from differentiating *Tie1*-EGFP embryonic stem cells with integrated puromycin resistance marker by using hygromycin selection (Marchetti *et al.*, 2002). Furthermore, as *Tie1*-Z/EGFP cells could be expanded in culture after FACS sorting, the first two possibilities are unlikely.

Tie1 is expressed in ECs of certain human tumors, suggesting that Tie1 is needed for tumor angiogenesis (Brown *et al.*, 2000; Hatva *et al.*, 1996; Hatva *et al.*, 1995; Kaipainen *et al.*, 1994; Lin *et al.*, 1999; Salven *et al.*, 1996). As the regulatory regions driving gene expression specifically in the angiogenic ECs may have potential for use in gene therapy, we analyzed the regulation of *Tie1* transcription during tumor neovascularization by transplanting Lewis lung carcinoma cells into *Tie1*-Z/EGFP, *Tie1*-LacZ or *Tie1+/LacZ* mice. Our results indicate that although endogenous *Tie1* transcription is activated in tumor endothelium, the mouse *Tie1* promoter fragment used did not contain the regulatory elements needed for the expression in tumor vasculature as no fluorescence or ß-galactosidase activity was seen in tumors grown in *Tie1*-Z/EGFP or *Tie1*-LacZ mice.

3. The genomic structure and promoter region of *VEGFR3* (III)

The coding sequence of the *VEGFR3* gene is organized into 31 exons that closely correspond to the genomic organization of the mouse *Vegfr1* and human *VEGFR2* genes (Kondo *et al.*, 1998; Yin *et al.*, 1998) (Fig. 8A).

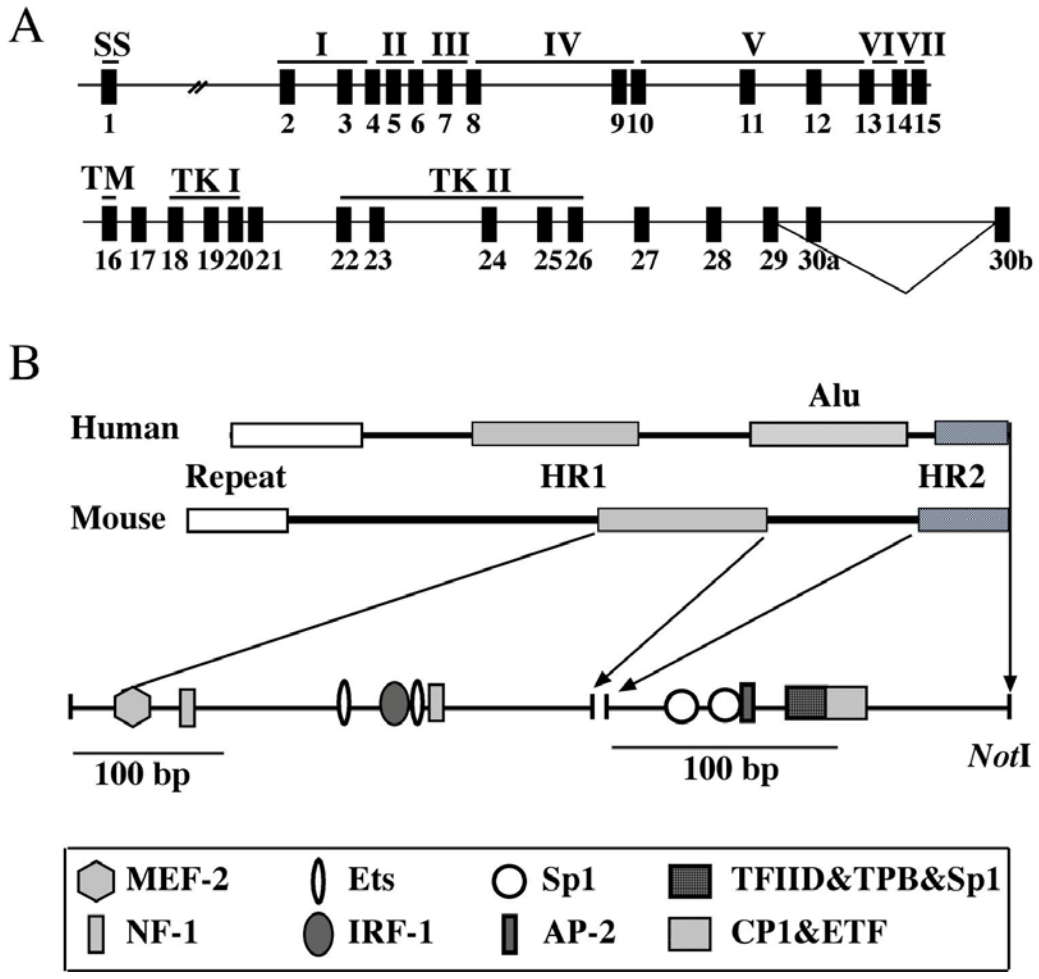

Figure 8. Exon-intron structure of the human *VEGFR3* and comparison of mouse and human *VEGFR3* promoters. (A) The genomic structure of the human *VEGFR3.* The exons encoding for signal sequence (SS), Ig-homology domains (I-VII), transmembrane region (TM) and tyrosine kinase domains (TK I and TK II) are indicated. (B) Schematic structure of the 5´ flanking regions of the human and mouse *VEGFR3* genes. The putative transcription factor binding sites present within the homology regions 1 and 2 are indicated. Adapted from Iljin *et al*., 2001.

All exon-intron boundaries were found to conform to the 5´-GT-AG-3´ splice site rule. The last two exons 30a and 30b of the *VEGFR3* are alternatively spliced. In mice, only the longer isoform exists, and it has been reported that the shorter transcript in humans is the result of a retroviral insertion (Hughes, 2001; Pajusola *et al.*, 1993). This insertion leads to diversity in the function of VEGFR-3, as the shorter form lacks three tyrosine residues that are important in downstream signaling (Fournier *et al.*, 1995; Fournier *et al.*, 1996). Resequencing of *VEGFR3* identified 22 intragenic single nucleotide polymorphisms and an intronic (CA)n repeat. The data concerning the exon-intron boundaries and polymorphisms of *VEGFR3* is clinically important and useful, as missense mutations in this gene have been linked to human hereditary lymphedema (Irrthum *et al.*, 2000; Karkkainen *et al.*, 2000). By using information about the *VEGFR3* genomic structure, rapid screening for mutations in *VEGFR3*-linked families of lymphedema or other inherited abnormalities of the lymphatic system is possible.

The 5' flanking regions of both human and mouse *VEGFR3* genes were analyzed and two homology regions (HR1 and HR2) showing 70% identity were identified (Fig. 8B). In the human promoter, these elements were separated by an Alu repeat. Similar arrangements of Alu sequences are located 5´ to the promoters of *PECAM-1*, *VWF* and *ENG,* and it has been suggested that a minimal promoter region would exist between the Alu sequence and the transcription initiation site (Almendro *et al.*, 1996; Assouline *et al.*, 1988; Gumina *et al.*, 1997; Ríus *et al.*, 1998). No classical TATA or CAAT boxes were found in the *VEGFR3* genes. The 3.0 kbp flanking fragment of *Vegfr3* demonstrated activity in cultured ECs but only minimal activity in keratinocytes and fibroblasts. Deletion studies of the *Vegfr3* promoter indicated that both homology elements were needed for activity. The 5´ homology element (HR1) had conserved putative binding sites for transcription factors conforming to the MEF-2, NF-1, AP-2, GATA and Ets families. Two EBSs were conserved in mouse and human promoters. The 3´ homology region contained multiple conserved binding sites for Sp1-like factors. Sp1 is a ubiquitous nuclear protein that can initiate transcription of TATA-less genes. Further studies are needed to determine which consensus binding sites are critical for the *Vegfr3* promoter activity.

To analyze whether the *Vegfr3* regulatory region identified *in vitro* is also functional *in vivo*, transgenic mouse embryos having either a 3.6 kbp *Hind*III /*Not*I, 1.6 kbp *Bsa*I/*Not*I or 0.8 kbp *Spe*I/*Not*I promoter fragment driving the expression of a *LacZ* reporter gene were generated. The expression of the reporters in transgenic embryos was compared to that of *Vegfr3+/LacZ* mice at E15.5, when the expression pattern is restricted to lymphatic endothelium. The 3.6 and 1.6 kbp *Vegfr3* promoter fragments directed weak lymphatic endothelial specific expression of the *LacZ* marker gene, whereas the 0.8 kbp promoter directed reporter expression also in the blood vessels, indicating that regulatory elements needed to restrict gene expression to the lymphatic endothelium are located within the 0.8 kbp *Bsa*I/*Spe*I *Vegfr3* promoter fragment.

The isolation of *Vegfr3* promoter makes it possible to identify transcription fac-

tors that contribute to lymphatic development using techniques of mouse molecular genetics. However, *Vegfr3* promoter driven reporter gene expression appeared to be very integration-site dependent. Therefore, for further studies, integration of the *Vegfr3* promoter driven transgene to a predetermined site in the genome by homologous recombination might be a better approach to generate transgenic mice than random integration generally used in transgenic assays. Recently, several studies on endothelial promoter-reporter gene constructs have successfully utilized this approach (Evans *et al.*, 2000; Guillot *et al.*, 2000). Also, isolation of putative additional enhancer elements present elsewhere in the *Vegfr3* locus might increase the efficiency of *Vegfr3* promoter driven transcription. Many tissue-specific genes have enhancer elements within the first two introns. *Tie2* and *Vegfr2* are examples of EC-specific genes whose activity is partly regulated by enhancers located in their first introns (Kappel *et al.*, 1999; Schlaeger *et al.*, 1997). Therefore, we started to analyze the first intron of *Vegfr3* consisting of 14.8 kbp of genomic DNA. When compared with the human *VEGFR3* first intronic sequence available, sequences were found to be 70% homologous. Several short regions showing high homology were found, but so far, no additional enhancers could be identified. Another way to increase the efficacy of *Vegfr3* promoter is to use "general" enhancers increasing promoter activity without losing its cellular specificity. This method has been used successfully with the EC-specific *VWF* promoter, suggesting that in combination with the 1.6 kbp *Vegfr3* promoter, efficient targeting to lymphatic vasculature after midgestation might be possible (Nettelbeck *et al.*, 1998).

4. Future prospects

Although our results suggest that NERF-2 regulates *Tie1* transcription *in vivo*, the direct evidence is still missing. It is possible that the transactivation seen after overexpression of the transcription factor is the result of indirect regulation due to the altered gene expression profile overall. Also, it is not certain that the genes regulated by a factor that is highly overexpressed as compared to its endogenous counterpart are true target genes. Therefore, to determine if NERF-2 binds to the endogenous *Tie1* promoter in physiologically relevant conditions, chromatin immunoprecipitation (ChIP) assay with cultured ECs could be performed. In this method, cells are briefly treated with formaldehyde to crosslink proteins to DNA. ChIP with an antibody against NERF-2, followed by PCR amplification using primers for the *Tie1* promoter, could reveal whether the *Tie1* promoter is a direct target of NERF-2 *in vivo*. To find out if NERF-2 regulates *Tie1* transcription during embryonic development, fluorescent ECs could be isolated from *Tie1*-Z/EGFP embryos by FACS, followed by the ChIP assay. Furthermore, by using a modification of the ChIP method, it is possible to clone individual promoter or enhancer fragments bound by a specific transcription factor and to identify transcriptional regulatory networks (Weinmann and Farnham, 2002). It would be interesting to study which regulatory elements are immunoprecipitated with NERF-2 antibody, and compare those with the ones immunoprecipitated with other antibodies, such as antibodies against Ets1.

Our results with both *Tie1* and *Vegfr3* promoters indicate that some regulatory elements needed to recapitulate the endogenous expression patterns of these genes are not present within the proximal promoter. To locate distant regulatory elements within the *Tie1* and *Vegfr3* genes and surrounding chromosomal regions, DNaseI sensitivity mapping or ChIP assays using antibodies specific for modified histones could be used (Johnson and Bresnick, 2002). Also, to find out which of the putative transcription factor binding sites present within HR1 and HR2 of the *Vegfr3* promoter are critical for promoter activity, site-directed mutagenesis followed by analysis of the mutant promoters in cultured cells could be performed. The results obtained with other EC-specific genes suggest that Ets and GATA sites might be important for *Vegfr3* promoter activity (for discussion, see Review of the literature; Regulation of EC-specific expression of genes and Results; DNA elements needed for the *Tie1* promoter activity and cell type specificity). The putative MEF-2 binding site may also be a critical DNA element for *Vegfr3* promoter activity, as mice with a targeted mutagenesis of MEF-2C transcription factor have a similar phenotype to VEGFR-3 deficient mice (Dumont *et al.,* 1998; Lin *et al.,* 1998). It would be certainly interesting to analyze the transcription driven by mutant promoters also in transgenic mice, but without the use of additional enhancers, it would be very challenging due to the weak and integration site dependent *Vegfr3* promoter activity *in vivo*.

CONCLUDING REMARKS

Although the formation of a functional vascular system is the result of a complex process involving multiple cell types, the initial formation of a vascular network is accomplished by endothelial cells alone. Results obtained from studies on EC-specific promoters may provide insights into the fundamental mechanisms that mediate EC-specific transcription.

A previous study had shown that the 0.8 kbp fragment of the mouse *Tie1* promoter is sufficient to drive EC-specific transcription of the *LacZ* reporter gene *in vivo*. We determined the critical DNA elements present within this region for the *Tie1* promoter activity. Our results indicate that Ets transcription factors are important regulators of *Tie1* promoter activity. Ets factors control the expression of many other EC-specific genes as well, suggesting that these transcription factors have an important role in vascular development. Among the Ets factors tested, NERF-2 was the most potent activator of the *Tie1* promoter. However, further studies are needed to determine whether it is the major transcription factor regulating *Tie1* expression during vascular development. We show that an octameric sequence and AP-2 site are also important for *Tie1* promoter activity. Our results with the *Tie1* promoter in combination with the results obtained from other EC-specific promoter studies indicate that the expression of EC-specific genes requires the combinatorial function of many transcription factors. Although the transcriptional regulation of EC-specific genes has common characteristics, it is evident that there are also differences, as the temporal and spatial expression pattern of different genes varies. In order to understand the transcriptional mechanisms underlying the specification of endothelial cells, precise determination of the expression patterns of putative transcription factors and target genes needs to be accomplished.

The promoter elements controlling endothelial-specific gene expression are of special interest because they may be useful in generating targeting vectors for gene therapy, directed to angiogenesis associated with tumor growth and metastasis, or other disease processes involving the vascular system. Our results indicate that the 0.8 kbp *Tie1* promoter is not suitable for targeting tumor endothelium but it can be used to drive the secretion of soluble proteins to the blood circulation and may therefore have potential for use in some applications of gene therapy.

Large-scale isolation of primary endothelial cells is of great interest for vascular gene profiling, for isolation of endothelial cells for tissue engineering, and for proangiogenic treatment of pathological conditions, such as tissue ischemia. Our results indicate that ECs can be isolated from *Tie1*-Z/EGFP transgenic mouse embryos by flow cytometry using EGFP. We believe that the *Tie1*-Z/EGFP mice could prove to be useful in angiogenesis research, as they can be mated with other transgenic or knock-out mice and the endothelial cells from the double transgenics can be easily isolated for further characterization *in vitro*.

As VEGFR-3 is specifically expressed on the lymphatic endothelium, the regu-

latory region of *VEGFR3* may provide an important molecular tool in the attempt to target the lymphatic system. In hereditary lymphedema with reduced VEGFR-3 signaling in heterozygous affected individuals, genes that induce VEGFR-3 signaling specifically in the lymphatic endothelium might improve the growth and function of lymphatic vessels without side effects in other non-target tissues.

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